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MULTIVALENT VACCINE FOR *CLOSTRIDIUM BOTULINUM* NEUROTOXIN

This application is a Continuation-In-Part of copending Application Serial No. 08/405,496, filed March 16, 1995.

FIELD OF THE INVENTION

5 The present invention relates to the isolation of polypeptides derived from *Clostridium botulinum* neurotoxins and the use thereof as immunogens for the production of vaccines, including multivalent vaccines, and antitoxins.

BACKGROUND OF THE INVENTION

10 The genus *Clostridium* is comprised of gram-positive, anaerobic, spore-forming bacilli. The natural habitat of these organisms is the environment and the intestinal tracts of humans and other animals. Indeed, clostridia are ubiquitous; they are commonly found in soil, dust, sewage, marine sediments, decaying vegetation, and mud. [See e.g., P.H.A. Sneath *et al.*, "*Clostridium*," *Bergey's Manual® of Systematic Bacteriology*, Vol. 2, pp. 1141-1200, Williams & Wilkins (1986).] Despite the
15 identification of approximately 100 species of *Clostridium*, only a small number have been recognized as etiologic agents of medical and veterinary importance. Nonetheless, these species are associated with very serious diseases, including botulism, tetanus, anaerobic cellulitis, gas gangrene, bacteremia, pseudomembranous colitis, and clostridial gastroenteritis. Table I lists some of the species of medical and
20 veterinary importance and the diseases with which they are associated. As virtually all of these species have been isolated from fecal samples of apparently healthy persons, some of these isolates may be transient, rather than permanent residents of the colonic flora.

TABLE 1

Clostridium Species Of Medical And Veterinary Importance*

Species	Disease
<i>C. aminovalericum</i>	Bacteriuria (pregnant women)
<i>C. argentinense</i>	Infected wounds; Bacteremia; Botulism; Infections of amniotic fluid
<i>C. baratii</i>	Infected war wounds; Peritonitis; Infectious processes of the eye, ear and prostate
<i>C. beijerinckii</i>	Infected wounds
<i>C. bifermentans</i>	Infected wounds; Abscesses; Gas Gangrene; Bacteremia
<i>C. botulinum</i>	Food poisoning; Botulism (wound, food, infant)
<i>C. butyricum</i>	Urinary tract, lower respiratory tract, pleural cavity, and abdominal infections; Infected wounds; Abscesses; Bacteremia
<i>C. cadaveris</i>	Abscesses; Infected wounds
<i>C. carnis</i>	Soft tissue infections; Bacteremia
<i>C. chauvoei</i>	Blackleg
<i>C. clostridioforme</i>	Abdominal, cervical, scrotal, pleural, and other infections; Septicemia; Peritonitis; Appendicitis
<i>C. cochlearium</i>	Isolated from human disease processes, but role in disease unknown.
<i>C. difficile</i>	Antimicrobial-associated diarrhea; Pseudomembranous enterocolitis; Bacteremia; Pyogenic infections
<i>C. fallax</i>	Soft tissue infections
<i>C. ghonii</i>	Soft tissue infections
<i>C. glycolicum</i>	Wound infections; Abscesses; Peritonitis
<i>C. hastiforme</i>	Infected war wounds; Bacteremia; Abscesses
<i>C. histolyticum</i>	Infected war wounds; Gas gangrene; Gingival plaque isolate
<i>C. indolis</i>	Gastrointestinal tract infections
<i>C. innocuum</i>	Gastrointestinal tract infections; Empyema
<i>C. irregulare</i>	Penile lesions
<i>C. leptum</i>	Isolated from human disease processes, but role in disease unknown.
<i>C. limosum</i>	Bacteremia; Peritonitis; Pulmonary infections

TABLE I

Clostridium Species Of Medical And Veterinary Importance*

Species	Disease
<i>C. malenominatum</i>	Various infectious processes
<i>C. novyi</i>	Infected wounds; Gas gangrene; Blackleg, Big head (ovine); Redwater disease (bovine)
<i>C. oroticum</i>	Urinary tract infections; Rectal abscesses
<i>C. paraputrificum</i>	Bacteremia; Peritonitis; Infected wounds; Appendicitis
<i>C. perfringens</i>	Gas gangrene; Anaerobic cellulitis; Intra-abdominal abscesses; Soft tissue infections; Food poisoning; Necrotizing pneumonia; Empyema; Meningitis; Bacteremia; Uterine Infections; Enteritis necrotans; Lamb dysentery; Struck; Ovine Enterotoxemia;
<i>C. putrefaciens</i>	Bacteriuria (Pregnant women with bacteremia)
<i>C. putrificum</i>	Abscesses; Infected wounds; Bacteremia
<i>C. ramosum</i>	Infections of the abdominal cavity, genital tract, lung, and biliary tract; Bacteremia
<i>C. sartagoforme</i>	Isolated from human disease processes, but role in disease unknown.
<i>C. septicum</i>	Gas gangrene; Bacteremia; Suppurative infections; Necrotizing enterocolitis; Braxy
<i>C. sordellii</i>	Gas gangrene; Wound infections; Penile lesions; Bacteremia; Abscesses; Abdominal and vaginal infections
<i>C. sphenoides</i>	Appendicitis; Bacteremia; Bone and soft tissue infections; Intraperitoneal infections; Infected war wounds; Visceral gas gangrene; Renal abscesses
<i>C. sporogenes</i>	Gas gangrene; Bacteremia; Endocarditis; central nervous system and pleuropulmonary infections; Penile lesions; Infected war wounds; Other pyogenic infections
<i>C. subterminale</i>	Bacteremia; Empyema; Biliary tract, soft tissue and bone infections
<i>C. symbiosum</i>	Liver abscesses; Bacteremia; Infections resulting due to bowel flora
<i>C. tertium</i>	Gas gangrene; Appendicitis; Brain abscesses; Intestinal tract and soft tissue infections; Infected war wounds; Periodontitis; Bacteremia

TABLE 1

Clostridium Species Of Medical And Veterinary Importance*

Species	Disease
<i>C. tetani</i>	Tetanus; Infected gums and teeth; Corneal ulcerations; Mastoid and middle ear infections; Intrapertitoneal infections; Tetanus neonatorum; Postpartum uterine infections; Soft tissue infections, especially related to trauma (including abrasions and lacerations); Infections related to use of contaminated needles
<i>C. thermosaccharolyticum</i>	Isolated from human disease processes, but role in disease unknown.

- * Compiled from P.G. Engelkirk *et al.* "Classification", *Principles and Practice of Clinical Anaerobic Bacteriology*, pp. 22-23, Star Publishing Co., Belmont, CA (1992); J. Stephen and R.A. Petrowski, "Toxins Which Traverse Membranes and Deregulate Cells," in *Bacterial Toxins*, 2d ed., pp. 66-67, American Society for Microbiology (1986); R. Berkow and A.J. Fletcher (eds.), "Bacterial Diseases," *Merck Manual of Diagnosis and Therapy*, 16th ed., pp. 116-126, Merck Research Laboratories, Rahway, N.J. (1992); and O.H. Sigmund and C.M. Fraser (eds.), "Clostridial Infections," *Merck Veterinary Manual*, 5th ed., pp. 396-409, Merck & Co., Rahway, N.J. (1979).

In most cases, the pathogenicity of these organisms is related to the release of powerful exotoxins or highly destructive enzymes. Indeed, several species of the genus *Clostridium* produce toxins and other enzymes of great medical and veterinary significance. [C.L. Hatheway, Clin. Microbiol. Rev. 3:66-98 (1990).]

Perhaps because of their significance for human and veterinary medicine, much research has been conducted on these toxins, in particular those of *C. botulinum* and *C. difficile*.

C. botulinum

Several strains of *Clostridium botulinum* produce toxins of significance to human and animal health. [C.L. Hatheway, Clin. Microbiol. Rev. 3:66-98 (1990)] The effects of these toxins range from diarrheal diseases that can cause destruction of the colon, to paralytic effects that can cause death. Particularly at risk for developing clostridial diseases are neonates and humans and animals in poor health (e.g., those suffering from diseases associated with old age or immunodeficiency diseases).

Clostridium botulinum produces the most poisonous biological toxin known. The lethal human dose is a mere 10^{-9} mg/kg bodyweight for toxin in the bloodstream. Botulinal toxin blocks nerve transmission to the muscles, resulting in flaccid paralysis. When the toxin reaches airway and respiratory muscles, it results in respiratory failure that can cause death. [S. Arnon, J. Infect. Dis. 154:201-206 (1986)]

C. botulinum spores are carried by dust and are found on vegetables taken from the soil, on fresh fruits, and on agricultural products such as honey. Under conditions favorable to the organism, the spores germinate to vegetative cells which produces toxin. [S. Arnon, Ann. Rev. Med. 31:541 (1980)]

Botulism disease may be grouped into four types, based on the method of introduction of toxin into the bloodstream. Food-borne botulism results from ingesting improperly preserved and inadequately heated food that contains botulinal toxin.

There were 355 cases of food-borne botulism in the United States between 1976 and 1984. [K.L. MacDonald *et al.*, Am. J. Epidemiol. 124:794 (1986).] The death rate due to botulinal toxin is 12% and can be higher in particular risk groups.

[C.O. Tacket *et al.*, Am. J. Med. 76:794 (1984).] Wound-induced botulism results from *C. botulinum* penetrating traumatized tissue and producing toxin that is absorbed into the bloodstream. Since 1950, thirty cases of wound botulism have been reported.

[M.N. Swartz, "Anaerobic Spore-Forming Bacilli: The Clostridia," pp. 633-646, in B.D. Davis *et al.*, (eds.), *Microbiology*, 4th edition, J.B. Lippincott Co. (1990).]

Inhalation botulism results when the toxin is inhaled. Inhalation botulism has been reported as the result of accidental exposure in the laboratory [E. Holzer, Med. Klin. 41:1735 (1962)] and could arise if the toxin is used as an agent of biological warfare

[D.R. Franz *et al.*, in *Botulinum and Tetanus Neurotoxins*, B.R. DasGupta, ed., Plenum Press, New York (1993), pp. 473-476]. Infectious infant botulism results from *C.*

botulinum colonization of the infant intestine with production of toxin and its absorption into the bloodstream. It is likely that the bacterium gains entry when

spores are ingested and subsequently germinate. [S. Arnon, J. Infect. Dis. 154:201 (1986).] There have been 500 cases reported since it was first recognized in 1976. [M.N. Swartz, *supra*.]

5 Infant botulism strikes infants who are three weeks to eleven months old (greater than 90% of the cases are infants less than six months). [S. Arnon, J. Infect. Dis. 154:201 (1986).] It is believed that infants are susceptible, due, in large part, to the absence of the full adult complement of intestinal microflora. The benign microflora present in the adult intestine provide an acidic environment that is not favorable to colonization by *C. botulinum*. Infants begin life with a sterile intestine
10 which is gradually colonized by microflora. Because of the limited microflora present in early infancy, the intestinal environment is not as acidic, allowing for *C. botulinum* spore germination, growth, and toxin production. In this regard, some adults who have undergone antibiotic therapy which alters intestinal microflora become more susceptible to botulism.

15 An additional factor accounting for infant susceptibility to infectious botulism is the immaturity of the infant immune system. The mature immune system is sensitized to bacterial antigens and produces protective antibodies. Secretory IgA produced in the adult intestine has the ability to agglutinate vegetative cells of *C. botulinum*. [S. Arnon, J. Infect. Dis. 154:201 (1986).] Secretory IgA may also act
20 by preventing intestinal bacteria and their products from crossing the cells of the intestine. [S. Arnon, Epidemiol. Rev. 3:45 (1981).] The infant immune system is not primed to do this.

Clinical symptoms of infant botulism range from mild paralysis, to moderate and severe paralysis requiring hospitalization, to fulminant paralysis, leading to sudden
25 death. [S. Arnon, Epidemiol. Rev. 3:45 (1981).]

The chief therapy for severe infant botulism is ventilatory assistance using a mechanical respirator and concurrent elimination of toxin and bacteria using cathartics,

enemas, and gastric lavage. There were 68 hospitalizations in California for infant botulism in a single year with a total cost of over \$4 million for treatment. [T.L. Frankovich and S. Arnon, West. J. Med. 154:103 (1991).]

5 Different strains of *Clostridium botulinum* each produce antigenically distinct toxin designated by the letters A-G. Serotype A toxin has been implicated in 26% of the cases of food botulism; types B, E and F have also been implicated in a smaller percentage of the food botulism cases [H. Sugiyama, Microbiol. Rev. 44:419 (1980)]. Wound botulism has been reportedly caused by only types A or B toxins [H. Sugiyama, *supra*]. Nearly all cases of infant botulism have been caused by bacteria
10 producing either type A or type B toxin. (Exceptionally, one New Mexico case was caused by *Clostridium botulinum* producing type F toxin and another by *Clostridium botulinum* producing a type B-type F hybrid.) [S. Arnon, Epidemiol. Rev. 3:45 (1981).] Type C toxin affects waterfowl, cattle, horses and mink. Type D toxin affects cattle, and type E toxin affects both humans and birds.

15 A trivalent antitoxin derived from horse plasma is commercially available from Connaught Industries Ltd. as a therapy for toxin types A, B, and E. However, the antitoxin has several disadvantages. First, extremely large dosages must be injected intravenously and/or intramuscularly. Second, the antitoxin has serious side effects such as acute anaphylaxis which can lead to death, and serum sickness. Finally, the
20 efficacy of the antitoxin is uncertain and the treatment is costly. [C.O. Tacket *et al.*, Am. J. Med. 76:794 (1984).]

A heptavalent equine botulinal antitoxin which uses only the F(ab')₂ portion of the antibody molecule has been tested by the United States Military. [M. Balady, USAMRDC Newsletter, p. 6 (1991).] This was raised against impure toxoids in those
25 large animals and is not a high titer preparation.

A pentavalent human antitoxin has been collected from immunized human subjects for use as a treatment for infant botulism. The supply of this antitoxin is

limited and cannot be expected to meet the needs of all individuals stricken with botulism disease. In addition, collection of human sera must involve screening out HIV and other potentially serious human pathogens. [P.J. Schwarz and S.S. Arnon, Western J. Med. 156:197 (1992).]

5 Infant botulism has been implicated as the cause of mortality in some cases of Sudden Infant Death Syndrome (SIDS, also known as crib death). SIDS is officially recognized as infant death that is sudden and unexpected and that remained unexplained despite complete post-mortem examination. The link of SIDS to infant botulism came when fecal or blood specimens taken at autopsy from SIDS infants
10 were found to contain *C. botulinum* organisms and/or toxin in 3-4% of cases analyzed. [D.R. Peterson *et al.*, Rev. Infect. Dis. 1:630 (1979).] In contrast, only 1 of 160 healthy infants (0.6%) had *C. botulinum* organisms in the feces and no botulinal toxin. (S. Arnon *et al.*, Lancet, pp. 1273-76, June 17, 1978.)

15 In developed countries, SIDS is the number one cause of death in children between one month and one year old. (S. Arnon *et al.*, Lancet, pp. 1273-77, June 17, 1978.) More children die from SIDS in the first year than from any other single cause of death in the first fourteen years of life. In the United States, there are 8,000-10,000 SIDS victims annually. *Id.*

20 What is needed is an effective therapy against infant botulism that is free of dangerous side effects, is available in large supply at a reasonable price, and can be safely and gently delivered so that prophylactic application to infants is feasible.

25 Immunization of subjects with toxin preparations has been done in an attempt to induce immunity against botulinal toxins. A *C. botulinum* vaccine comprising chemically inactivated (*i.e.*, formaldehyde-treated) type A, B, C, D and E toxin is commercially available for human usage. However, this vaccine preparation has several disadvantages. First, the efficacy of this vaccine is variable (in particular, only 78% of recipients produce protective levels of anti-type B antibodies following

administration of the primary series). Second, immunization is painful (deep subcutaneous inoculation is required for administration), with adverse reactions being common (moderate to severe local reactions occur in approximately 6% of recipients upon initial injection; this number rises to approximately 11% of individuals who receive booster injections) [Informational Brochure for the Pentavalent (ABCDE) Botulinum Toxoid, Centers for Disease Control]. Third, preparation of the vaccine is dangerous as active toxin must be handled by laboratory workers.

What is needed are safe and effective vaccine preparations for administration to those at risk of exposure to *C. botulinum* toxins.

C. difficile

C. difficile, an organism which gained its name due to difficulties encountered in its isolation, has recently been proven to be an etiologic agent of diarrheal disease. (Sneath *et al.*, p. 1165.). *C. difficile* is present in the gastrointestinal tract of approximately 3% of healthy adults, and 10-30% of neonates without adverse effect (Swartz, at p. 644); by other estimates, *C. difficile* is a part of the normal gastrointestinal flora of 2-10% of humans. [G.F. Brooks *et al.*, (eds.) "*Infections Caused by Anaerobic Bacteria*," Jawetz, Melnick, & Adelberg's *Medical Microbiology*, 19th ed., pp. 257-262, Appleton & Lange, San Mateo, CA (1991).] As these organisms are relatively resistant to most commonly used antimicrobials, when a patient is treated with antibiotics, the other members of the normal gastrointestinal flora are suppressed and *C. difficile* flourishes, producing cytopathic toxins and enterotoxins. It has been found in 25% of cases of moderate diarrhea resulting from treatment with antibiotics, especially the cephalosporins, clindamycin, and ampicillin. [M.N. Swartz at 644.]

Importantly, *C. difficile* is commonly associated with nosocomial infections. The organism is often present in the hospital and nursing home environments and may

be carried on the hands and clothing of hospital personnel who care for debilitated and immunocompromised patients. As many of these patients are being treated with antimicrobials or other chemotherapeutic agents, such transmission of *C. difficile* represents a significant risk factor for disease. (Engelkirk *et al.*, pp. 64-67.)

5 *C. difficile* is associated with a range of diarrhetic illness, ranging from diarrhea alone to marked diarrhea and necrosis of the gastrointestinal mucosa with the accumulation of inflammatory cells and fibrin, which forms a pseudomembrane in the affected area. (Brooks *et al.*) It has been found in over 95% of pseudomembranous enterocolitis cases. (Swartz, at p. 644.) This occasionally fatal disease is characterized
10 by diarrhea, multiple small colonic plaques, and toxic megacolon. (Swartz, at p. 644.) Although stool cultures are sometimes used for diagnosis, diagnosis is best made by detection of the heat labile toxins present in fecal filtrates from patients with enterocolitis due to *C. difficile*. (Swartz, at p. 644-645; and Brooks *et al.*, at p. 260.) *C. difficile* toxins are cytotoxic for tissue/cell cultures and cause enterocolitis when
15 injected intracecally into hamsters. (Swartz, at p. 644.)

The enterotoxicity of *C. difficile* is primarily due to the action of two toxins, designated A and B, each of approximately 300,000 in molecular weight. Both are potent cytotoxins, with toxin A possessing direct enterocytotoxic activity. [Lyerly *et al.*, Infect. Immun. 60:4633 (1992).] Unlike toxin A of *C. perfringens*, an organism
20 rarely associated with antimicrobial-associated diarrhea, the toxin of *C. difficile* is not a spore coat constituent and is not produced during sporulation. (Swartz, at p. 644.) *C. difficile* toxin A causes hemorrhage, fluid accumulation and mucosal damage in rabbit ileal loops and appears to increase the uptake of toxin B by the intestinal mucosa. Toxin B does not cause intestinal fluid accumulation, but it is 1000 times
25 more toxic than toxin A to tissue culture cells and causes membrane damage. Although both toxins induce similar cellular effects such as actin disaggregation, differences in cell specificity occurs.

Both toxins are important in disease. [Borriello *et al.*, Rev. Infect. Dis., 12(suppl. 2):S185 (1990); Lysterly *et al.*, Infect. Immun., 47:349 (1985); and Rolfe, Infect. Immun., 59:1223 (1990).] Toxin A is thought to act first by binding to brush border receptors, destroying the outer mucosal layer, then allowing toxin B to gain access to the underlying tissue. These steps in pathogenesis would indicate that the production of neutralizing antibodies against toxin A may be sufficient in the prophylactic therapy of CDAD. However, antibodies against toxin B may be a necessary additional component for an effective therapeutic against later stage colonic disease. Indeed, it has been reported that animals require antibodies to both toxin A and toxin B to be completely protected against the disease. [Kim and Rolfe, Abstr. Ann. Meet. Am. Soc. Microbiol., 69:62 (1987).]

C. difficile has also been reported to produce other toxins such as an enterotoxin different from toxins A and B [Banno *et al.*, Rev. Infect. Dis., 6(Suppl. 1):S11-S20 (1984)], a low molecular weight toxin [Rihn *et al.*, Biochem. Biophys. Res. Comm., 124:690-695 (1984)], a motility altering factor [Justus *et al.*, Gastroenterol., 83:836-843 (1982)], and perhaps other toxins. Regardless, *C. difficile* gastrointestinal disease is of primary concern.

It is significant that due to its resistance to most commonly used antimicrobials, *C. difficile* is associated with antimicrobial therapy with virtually all antimicrobial agents (although most commonly ampicillin, clindamycin and cephalosporins). It is also associated with disease in patients undergoing chemotherapy with such compounds as methotrexate, 5-fluorouracil, cyclophosphamide, and doxorubicin. [S.M. Finegold *et al.*, *Clinical Guide to Anaerobic Infections*, pp. 88-89, Star Publishing Co., Belmont, CA (1992).]

Treatment of *C. difficile* disease is problematic, given the high resistance of the organism. Oral metronidazole, bacitracin and vancomycin have been reported to be effective. (Finegold *et al.*, p. 89.) However there are problems associated with

treatment utilizing these compounds. Vancomycin is very expensive, some patients are unable to take oral medication, and the relapse rate is high (20-25%), although it may not occur for several weeks. *Id.*

C. difficile disease would be prevented or treated by neutralizing the effects of these toxins in the gastrointestinal tract. Thus, what is needed is an effective therapy against *C. difficile* toxin that is free of dangerous side effects, is available in large supply at a reasonable price, and can be safely delivered so that prophylactic application to patients at risk of developing pseudomembranous enterocolitis can be effectively treated.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows the reactivity of anti-*C. botulinum* IgY by Western blot.

Figure 2 shows the IgY antibody titer to *C. botulinum* type A toxoid in eggs, measured by ELISA.

Figure 3 shows the results of *C. difficile* toxin A neutralization assays.

Figure 4 shows the results of *C. difficile* toxin B neutralization assays.

Figure 5 shows the results of *C. difficile* toxin B neutralization assays.

Figure 6 is a restriction map of *C. difficile* toxin A gene, showing sequences of primers 1-4 (SEQ ID NOS:1-4).

Figure 7 is a Western blot of *C. difficile* toxin A reactive protein.

Figure 8 shows *C. difficile* toxin A expression constructs.

Figure 9 shows *C. difficile* toxin A expression constructs.

Figure 10 shows the purification of recombinant *C. difficile* toxin A.

Figure 11 shows the results of *C. difficile* toxin A neutralization assays with antibodies reactive to recombinant toxin A.

Figure 12 shows the results for a *C. difficile* toxin A neutralization plate.

Figure 13 shows the results for a *C. difficile* toxin A neutralization plate.

Figure 14 shows the results of recombinant *C. difficile* toxin A neutralization assays.

Figure 15 shows *C. difficile* toxin A expression constructs.

5 Figure 16 shows a chromatograph plotting absorbance at 280 nm against retention time for a pMA1870-680 IgY PEG preparation.

Figure 17 shows two recombinant *C. difficile* toxin B expression constructs.

Figure 18 shows *C. difficile* toxin B expression constructs.

Figure 19 shows *C. difficile* toxin B expression constructs.

Figure 20 shows *C. difficile* toxin B expression constructs.

10 Figure 21 is an SDS-PAGE gel showing the purification of recombinant *C. difficile* toxin B fusion protein.

Figure 22 is an SDS-PAGE gel showing the purification of two histidine-tagged recombinant *C. difficile* toxin B proteins.

Figure 23 shows *C. difficile* toxin B expression constructs.

15 Figure 24 is a Western blot of *C. difficile* toxin B reactive protein.

Figure 25 shows *C. botulinum* type A toxin expression constructs; constructs used to provide *C. botulinum* or *C. difficile* sequences are also shown.

Figure 26 is an SDS-PAGE gel stained with Coomassie blue showing the purification of recombinant *C. botulinum* type A toxin fusion proteins.

20 Figure 27 shows *C. botulinum* type A toxin expression constructs; constructs used to provide *C. botulinum* sequences are also shown.

Figure 28 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBot protein using the Ni-NTA resin.

25 Figure 29 is an SDS-PAGE gel stained with Coomassie blue showing the expression of pHisBot protein in BL21(DE3) and BL21(DE3)pLysS host cells.

Figure 30 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBot protein using a batch absorption procedure.

Figure 31 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBot and pHisBot(native) proteins using a Ni-NTA column.

Figure 32 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBotA protein expressed in pHisBotA(syn) kan lacIq
5 T7/pACYCGro/BL21(DE3) cells using an IDA column.

Figure 33 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBotA, pHisBotB and pHisBotE proteins by IDA chromatography followed by chromatography on S-100 to remove folding chaperones.

Figure 34 is an SDS-PAGE gel stained with Coomassie blue showing the
10 extracts derived from pHisBotB amp T7lac/BL21(DE3) cells before and after purification on a Ni-NTA column.

Figure 35 is an SDS-PAGE gel run under native conditions and stained with Coomassie blue showing the removal of folding chaperones from IDA-purified BotB protein using a S-100 column.

Figure 36 is an SDS-PAGE gel stained with Coomassie blue showing proteins
15 that eluted during an imidazole step gradient applied to a IDA column containing a lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells.

Figure 37 is an SDS-PAGE gel run under native conditions and stained with Coomassie blue showing IDA-purified BotB protein before and after ultrafiltration.

Figure 38 is an SDS-PAGE gel stained with Coomassie blue showing the
20 purification of BotE protein using a NiNTA column.

Figure 39 is an SDS-PAGE gel stained with Coomassie blue showing extracts derived from pHisBotA kan T7 lac/BL21(DE3) pLysS cells grown in fermentation culture.

Figure 40 is a chromatogram showing proteins present after IDA-purified BotE
25 protein was applied to a S-100 column.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

5 As used herein, the term "neutralizing" is used in reference to antitoxins, particularly antitoxins comprising antibodies, which have the ability to prevent the pathological actions of the toxin against which the antitoxin is directed.

As used herein, the term "overproducing" is used in reference to the production of clostridial toxin polypeptides in a host cell and indicates that the host cell is producing more of the clostridial toxin by virtue of the introduction of nucleic acid
10 sequences encoding said clostridial toxin polypeptide than would be expressed by said host cell absent the introduction of said nucleic acid sequences. To allow ease of purification of toxin polypeptides produced in a host cell it is preferred that the host cell express or overproduce said toxin polypeptide at a level greater than 1 mg/liter of host cell culture.

15 "A host cell capable of expressing a recombinant protein at a level greater than or equal to 5% of the total cellular protein" is a host cell in which the recombinant protein represents at least 5% of the total cellular protein. To determine what percentage of total cellular protein the recombinant protein represents, the following steps are taken. A total of 10 OD₆₀₀ units of recombinant host cells (e.g., 200 µl of
20 cells at OD₆₀₀=50/ml) are removed (at a timepoint known to represent the peak of expression of the desired recombinant protein) to a 1.5 ml microfuge tube and pelleted for 2 min at maximum rpm in a microfuge. The pellets are resuspended in 1 ml of 50 mM NaHPO₄, 0.5 M NaCl, 40 mM imidazole buffer (pH 6.8) containing 1 mg/ml lysozyme. The samples are incubated for 20 min at room temperature and stored ON
25 at -70°C. Samples are thawed completely at room temperature and sonicated 2 X 10 seconds with a Branson Sonifier 450 microtip probe at # 3 power setting. The samples are centrifuged for 5 min. at maximum rpm in a microfuge. An aliquot (20

μl) of the protein sample is removed to 20 μl 2X sample buffer (this represents the total protein extract). The samples are heated to 95°C for 5 min, then cooled and 5 or 10 μl are loaded onto 12.5% SDS-PAGE gels. High molecular weight protein markers are also loaded to allow for estimation of the MW of identified recombinant proteins.

5 After electrophoresis, protein is detected generally by staining with Coomassie blue and the stained gel is scanned using a densitometer to determine the percentage of protein present in each band. In this manner, the percentage of protein present in the band corresponding to the recombinant protein of interest may be determined. It is not necessary that Coomassie blue be employed for the detection of protein, a number of
10 fluorescent dyes [e.g., Sypro orange S-6651 (Molecular Probes, Eugene, OR)] may be employed and the stained gel scanned using a fluoroimager [e.g., Fluor Imager SI (Molecular Dynamics, Sunnyvale, CA)].

"A host cell capable of expressing a recombinant protein as a soluble protein at a level greater than or equal to 0.25% of the total soluble cellular protein" is a host
15 cell in which the amount of soluble recombinant protein present represents at least 0.25% of the total cellular protein. As used herein "total soluble cellular protein" refers to a clarified PEI lysate prepared as described in Example 31(c)(iv). Briefly, cells are harvested following induction of expression of recombinant protein (at a point of maximal expression). The cells are resuspended in cell resuspension buffer (CRB:
20 50 mM NaPO₄, 0.5 M NaCl, 40 mM imidazole, pH 6.8) to create a 20% cell suspension (wet weight of cells/volume of CRB) and cell lysates are prepared as described in Example 31(c)(iv) (i.e., sonication or homogenization followed by centrifugation). The cell lysate is then flocculated utilizing polyethyleneimine (PEI) prior to centrifugation. PEI (a 2% solution in dH₂O, pH 7.5 with HCl) is added to the
25 cell lysate to a final concentration of 0.2%, and stirred for 20 min at room temperature prior to centrifugation [8,500 rpm in JA10 rotor (Beckman) for 30 minutes at 4°C]. This treatment removes RNA, DNA and cell wall components, resulting in a clarified,

low viscosity lysate ("PEI clarified lysate"). The recombinant protein present in the PEI clarified lysate is then purified (e.g., by chromatography on an IDA column for his-tagged proteins). The amount of purified recombinant protein (i.e., the eluted protein) is divided by the concentration of protein present in the PEI clarified lysate (typically 8 mg/ml when using a 20% cell suspension as the starting material) and multiplied by 100 to determine what percentage of total soluble cellular protein is comprised of the soluble recombinant protein (see Example 33b).

As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (i.e., *C. botulinum* toxin A, B, C, D, E, F, or G and fragments thereof) joined to an exogenous protein fragment (the fusion partner which consists of a non-toxin protein). The fusion partner may enhance solubility of the *C. botulinum* protein as expressed in a host cell, may provide an affinity tag to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both. If desired, the fusion protein may be removed from the protein of interest (i.e., toxin protein or fragments thereof) prior to immunization by a variety of enzymatic or chemical means known to the art.

As used herein the term "non-toxin protein" or "non-toxin protein sequence" refers to that portion of a fusion protein which comprises a protein or protein sequence which is not derived from a bacterial toxin protein.

The term "protein of interest" as used herein refers to the protein whose expression is desired within the fusion protein. In a fusion protein the protein of interest will be joined or fused with another protein or protein domain, the fusion partner, to allow for enhanced stability of the protein of interest and/or ease of purification of the fusion protein.

As used herein, the term "maltose binding protein" refers to the maltose binding protein of *E. coli*. A portion of the maltose binding protein may be added to a protein of interest to generate a fusion protein; a portion of the maltose binding protein may

merely enhance the solubility of the resulting fusion protein when expressed in a bacterial host. On the other hand, a portion of the maltose binding protein may allow affinity purification of the fusion protein on an amylose resin.

5 As used herein, the term "poly-histidine tract" when used in reference to a fusion protein refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus of a protein of interest. A poly-histidine tract of six to ten residues is preferred. The poly-histidine tract is also defined functionally as being a number of consecutive histidine residues added to the protein of interest which allows the affinity purification of the resulting fusion protein on a nickel-chelate or IDA
10 column.

As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, antitoxins are purified by removal of contaminating non-immunoglobulin proteins; they are also purified by the removal of immunoglobulin that does not bind toxin. The removal of non-immunoglobulin
15 proteins and/or the removal of immunoglobulins that do not bind toxin results in an increase in the percent of toxin-reactive immunoglobulins in the sample. In another example, recombinant toxin polypeptides are expressed in bacterial host cells and the toxin polypeptides are purified by the removal of host cell proteins; the percent of recombinant toxin polypeptides is thereby increased in the sample. Additionally, the
20 recombinant toxin polypeptides are purified by the removal of host cell components such as lipopolysaccharide (*e.g.*, endotoxin).

The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

25 The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

The term "native protein" as used herein refers to a protein which is isolated from a natural source as opposed to the production of a protein by recombinant means.

As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

As used herein "soluble" when in reference to a protein produced by recombinant DNA technology in a host cell is a protein which exists in solution in the cytoplasm of the host cell; if the protein contains a signal sequence the soluble protein is exported to the periplasmic space in bacteria hosts and is secreted into the culture medium in eucaryotic cells capable of secretion or by bacterial host possessing the appropriate genes (*i.e.*, the *kil* gene). In contrast, an insoluble protein is one which exists in denatured form inside cytoplasmic granules (called inclusion bodies) in the host cell. High level expression (*i.e.*, greater than 10-20 mg recombinant protein/liter of bacterial culture) of recombinant proteins often results in the expressed protein being found in inclusion bodies in the bacterial host cells. A soluble protein is a protein which is not found in an inclusion body inside the host cell or is found both in the cytoplasm and in inclusion bodies and in this case the protein may be present at high or low levels in the cytoplasm.

A distinction is drawn between a soluble protein (*i.e.*, a protein which when expressed in a host cell is produced in a soluble form) and a "solubilized" protein. An insoluble recombinant protein found inside an inclusion body may be solubilized (*i.e.*, rendered into a soluble form) by treating purified inclusion bodies with denaturants such as guanidine hydrochloride, urea or sodium dodecyl sulfate (SDS). These denaturants must then be removed from the solubilized protein preparation to allow the recovered protein to renature (refold). Not all proteins will refold into an active conformation after solubilization in a denaturant and removal of the denaturant. Many

proteins precipitate upon removal of the denaturant. SDS may be used to solubilize inclusion bodies and will maintain the proteins in solution at low concentration. However, dialysis will not always remove all of the SDS (SDS can form micelles which do not dialyze out); therefore, SDS-solubilized inclusion body protein is soluble but not refolded.

A distinction is drawn between proteins which are soluble (*i.e.*, dissolved) in a solution devoid of significant amounts of ionic detergents (*e.g.*, SDS) or denaturants (*e.g.*, urea, guanidine hydrochloride) and proteins which exist as a suspension of insoluble protein molecules dispersed within the solution. A soluble protein will not be removed from a solution containing the protein by centrifugation using conditions sufficient to remove bacteria present in a liquid medium (*i.e.*, centrifugation at 12,000 x g for 4-5 minutes). For example, to test whether two proteins, protein A and protein B, are soluble in solution, the two proteins are placed into a solution selected from the group consisting of PBS-NaCl (PBS containing 0.5 M NaCl), PBS-NaCl containing 0.2% Tween 20, PBS, PBS containing 0.2% Tween 20, PBS-C (PBS containing 2 mM CaCl_2), PBS-C containing either 0.1 or 0.5 % Tween 20, PBS-C containing either 0.1 or 0.5% NP-40, PBS-C containing either 0.1 or 0.5% Triton X-100, PBS-C containing 0.1% sodium deoxycholate. The mixture containing proteins A and B is then centrifuged at 5000 x g for 5 minutes. The supernatant and pellet formed by centrifugation are then assayed for the presence of protein A and B. If protein A is found in the supernatant and not in the pellet [except for minor amounts (*i.e.*, less than 10%) as a result of trapping], protein is said to be soluble in the solution tested. If the majority of protein B is found in the pellet (*i.e.*, greater than 90%), then protein B is said to exist as a suspension in the solution tested.

As used herein, the term "therapeutic amount" refers to that amount of antitoxin required to neutralize the pathologic effects of one or more clostridial toxins in a subject.

The term "pyrogen" as used herein refers to a fever-producing substance. Pyrogens may be endogenous to the host (*e.g.*, prostaglandins) or may be exogenous compounds (*e.g.*, bacterial endo- and exotoxins, nonbacterial compounds such as antigens and certain steroid compounds, etc.). The presence of pyrogen in a pharmaceutical solution may be detected using the U.S. Pharmacopeia (USP) rabbit fever test (United States Pharmacopeia, Vol. XXII (1990) United States Pharmacopeial Convention, Rockville, MD, p. 151).

The term "endotoxin" as used herein refers to the high molecular weight complexes associated with the outer membrane of gram-negative bacteria. Unpurified endotoxin contains lipids, proteins and carbohydrates. Highly purified endotoxin does not contain protein and is referred to as lipopolysaccharide (LPS). Because unpurified endotoxin is of concern in the production of pharmaceutical compounds (*e.g.*, proteins produced in *E. coli* using recombinant DNA technology), the term endotoxin as used herein refers to unpurified endotoxin. Bacterial endotoxin is a well known pyrogen.

As used herein, the term "endotoxin-free" when used in reference to a composition to be administered parenterally (with the exception of intrathecal administration) to a host means that the dose to be delivered contains less than 5 EU/kg body weight [FDA Guidelines for Parenteral Drugs (December 1987)]. Assuming a weight of 70 kg for an adult human, the dose must contain less than 350 EU to meet FDA Guidelines for parenteral administration. Endotoxin levels are measured herein using the Limulus Amebocyte Lysate (LAL) test (Limulus Amebocyte Lysate Pyrochrome™, Associates of Cape Cod, Inc. Woods Hole, MA). To measure endotoxin levels in preparations of recombinant proteins, 0.5 ml of a solution comprising 0.5 mg of purified recombinant protein in 50 mM NaPO₄, pH 7.0, 0.3M NaCl and 10% glycerol is used in the LAL assay according to the manufacturer's instructions for the endpoint chromogenic without diazo-coupling method [the specific components of the buffer containing recombinant protein to be analyzed in the LAL

test are not important; any buffer having a neutral pH may be employed (see for example, alternative buffers employed in Examples 34, 40 and 45)]. Compositions containing less than or equal to than 250 endotoxin units (EU)/mg of purified recombinant protein are herein defined as "substantially endotoxin-free." Preferably the composition contains less than or equal to 100, and most preferably less than or equal to 60, (EU)/mg of purified recombinant protein. Typically, administration of bacterial toxins or toxoids to adult humans for the purpose of vaccination involves doses of about 10-500 µg protein/dose. Therefore, administration of 10-500 µg of a purified recombinant protein to a 70 kg human, wherein said purified recombinant protein preparation contains 60 EU/mg protein, results in the introduction of only 0.6 to 30 EU (*i.e.*, 0.2 to 8.6% of the maximum allowable endotoxin burden per parenteral dose). Administration of 10-500 µg of a purified recombinant protein to a 70 kg human, wherein said purified recombinant protein preparation contains 250 EU/mg protein, results in the introduction of only 2.5 to 125 EU (*i.e.*, 0.7 to 36% of the maximum allowable endotoxin burden per parenteral dose).

The LAL test is accepted by the U.S. FDA as a means of detecting bacterial endotoxins (21 C.F.R. §§ 660.100 -105). Studies have shown that the LAL test is equivalent or superior to the USP rabbit pyrogen test for the detection of endotoxin and thus the LAL test can be used as a surrogate for pyrogenicity studies in animals [F.C. Perason, *Pyrogens: endotoxins, LAL testing and depyrogenation*, Marcel Dekker, New York (1985), pp.150-155]. The FDA Bureau of Biologics accepts the LAL assay in place of the USP rabbit pyrogen test so long as the LAL assay utilized is shown to be as sensitive as, or more sensitive as the rabbit test [Fed. Reg., 38, 26130 (1980)].

The term "monovalent" when used in reference to a clostridial vaccine refers to a vaccine which is capable of provoking an immune response in a host animal directed against a single type of clostridial toxin. For example, if immunization of a host with *C. botulinum* type A toxin vaccine induces antibodies in the immunized host which

protect against a challenge with type A toxin but not against challenge with type B, C, D, E, F or G toxins, then the type A vaccine is said to be monovalent. In contrast, a "multivalent" vaccine provokes an immune response in a host animal directed against several (*i.e.*, more than one) clostridial toxins. For example, if immunization of a host with a vaccine comprising *C. botulinum* type A and B toxins induces the production of antibodies which protect the host against a challenge with both type A and B toxin, the vaccine is said to be multivalent (in particular, this hypothetical vaccine is bivalent).

As used herein the term "immunogenically-effective amount" refers to that amount of an immunogen required to invoke the production of protective levels of antibodies in a host upon vaccination.

The term "protective level", when used in reference to the level of antibodies induced upon immunization of the host with an immunogen which comprises a bacterial toxin, means a level of circulating antibodies sufficient to protect the host from challenge with a lethal dose of the toxin.

As used herein the terms "protein" and "polypeptide" refer to compounds comprising amino acids joined via peptide bonds and are used interchangeably.

The terms "toxin" and "neurotoxin" when used in reference to toxins produced by members (*i.e.*, species and strains) of the genus *Clostridium* are used interchangeably and refer to the proteins which are poisonous to nerve tissue.

The term "receptor-binding domain" when used in reference to a *C. botulinum* toxin refers to the carboxy-terminal portion of the heavy chain (H_C or the C fragment) of the toxin which is presumed to be responsible for the binding of the active toxin (*i.e.*, the derivative toxin comprising the H and L chains joined via disulfide bonds) to receptors on the surface of synaptosomes. The receptor-binding domain for *C. botulinum* type A toxin is defined herein as comprising amino acid residues 861 through 1296 of SEQ ID NO:28. The receptor-binding domain for *C. botulinum* type B toxin is defined herein as comprising amino acid residues 848 through 1291 of SEQ

ID NO:40 (strain Eklund 17B). The receptor-binding domain of *C. botulinum* type C1 toxin is defined herein as comprising amino acid residues 856 through 1291 of SEQ ID NO:60. The receptor-binding domain of *C. botulinum* type D toxin is defined herein as comprising amino acid residues 852 through 1276 of SEQ ID NO:66. The
5 receptor-binding domain of *C. botulinum* type E toxin is defined herein as comprising amino acid residues 835 through 1250 of SEQ ID NO:50 (Beluga strain). The receptor-binding domain of *C. botulinum* type F toxin is defined herein as comprising amino acid residues 853 through 1274 of SEQ ID NO:71. The receptor-binding
10 domain of *C. botulinum* type G toxin is defined herein as comprising amino acid residues 853 through 1297 of SEQ ID NO:77. Within a given serotype, small variations in the primary amino acid sequence of the botulinal toxins isolated from different strains has been reported [Whelan *et al.* (1992), *supra* and Minton (1995) Curr. Top. Microbiol. Immunol. 195:161-194]. The present invention contemplates
15 fusion proteins comprising the receptor-binding domain of *C. botulinum* toxins from serotypes A-G including the variants found among different strains within a given serotype. The receptor-binding domains listed above are used as the prototype for each strain within a serotype. Fusion proteins containing an analogous region from a strain other than the prototype strain are encompassed by the present invention.

Fusion proteins comprising the receptor binding domain (*i.e.*, C fragment) of
20 botulinal toxins may include amino acid residues located beyond the termini of the domains defined above. For example, the pHisBotB protein contains amino acid residues 846-1291 of SEQ ID NO:40; this fusion protein thus comprises the receptor-binding domain for *C. botulinum* type B toxin as defined above (*i.e.*, Ile-848 through Glu-1291). Similarly, pHisBotE contains amino acid residues 827-1252 of SEQ ID
25 NO:50 and pHisBotG contains amino acid residues 851-1297 of SEQ ID NO:77. Thus, both pHisBotE and pHisBotG fusion proteins contain a few amino acids located beyond the N-terminus of the defined receptor-binding domain.

The terms "native gene" or "native gene sequences" are used to indicate DNA sequences encoding a particular gene which contain the same DNA sequences as found in the gene as isolated from nature. In contrast, "synthetic gene sequences" are DNA sequences which are used to replace the naturally occurring DNA sequences when the naturally occurring sequences cause expression problems in a given host cell. For example, naturally-occurring DNA sequences encoding codons which are rarely used in a host cell may be replaced (*e.g.*, by site-directed mutagenesis) such that the synthetic DNA sequence represents a more frequently used codon. The native DNA sequence and the synthetic DNA sequence will preferably encode the same amino acid sequence.

SUMMARY OF THE INVENTION

The present invention relates to the production of polypeptides derived from toxins particularly in recombinant host cells. In one embodiment, the present invention provides a host cell containing a recombinant expression vector, said vector encoding a protein comprising at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin. The present invention is not limited by the nature of sequences encoding portions of the *C. botulinum* toxin. These sequences may be derived from the native gene sequences or alternatively they may comprise synthetic gene sequences. Synthetic gene sequences are employed when expression of the native gene sequences is problematic in a given host cell (*e.g.*, when the native gene sequences contain sequences resembling yeast transcription termination signals and the desired host cell is a yeast cell).

In one embodiment, the host cell is capable of expressing the recombinant *C. botulinum* toxin protein at a level greater than or equal to 2% to 40% of the total cellular protein and preferably at a level greater than or equal to 5% of the total cellular protein. In another embodiment, the host cell is capable of expressing the recombinant *C. botulinum* toxin protein as a soluble protein at a level greater than or

equal to 0.25% of the total cellular protein and preferably at a level greater than or equal to 0.25% to 10% of the total cellular protein.

The present invention is not limited by the nature of the host cell employed for the production of recombinant *C. botulinum* toxin proteins. In a preferred
5 embodiment, the host cell is an *E. coli* cell. In another preferred embodiment, the host cell is an insect cell; particularly preferred insect host cells are *Spodoptera frugiperda* (Sf9) cells. In another preferred embodiment, the host cell is a yeast cell; particularly preferred yeast cells are *Pichia pastoris* cells.

In another embodiment, the invention provides a host cell containing a
10 recombinant expression vector, said vector encoding a fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin. The invention is not limited by the nature of the portion of the *Clostridium botulinum* toxin selected. In a preferred embodiment, the portion of the toxin comprises the receptor
15 binding domain (*i.e.*, the C fragment of the toxin). The present invention is not limited by the nature of the non-toxin protein sequence employed. In a preferred embodiment, the non-toxin protein sequence comprises a poly-histidine tract. A number of alternative fusion tags or fusion partners are known to the art (*e.g.*, MBP, GST, protein A, etc.) and may be employed for the production of fusion proteins
20 comprising a portion of a botulinal toxin.

The present invention further provides a vaccine comprising a fusion protein, said fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin. The vaccine may be a monovalent vaccine (*i.e.*, containing
25 only a toxin B fusion protein or a toxin E fusion protein), a bivalent vaccine (*i.e.*, containing both a toxin B fusion protein and a toxin E fusion protein) or a trivalent or higher valency vaccine. In a preferred embodiment, the toxin B fusion protein and/or

toxin E fusion protein is combined with a fusion protein comprising a non-toxin protein sequence and at least a portion of *Clostridium botulinum* type A toxin. The present invention is not limited by the nature of the portion of the *Clostridium botulinum* toxin selected. In a preferred embodiment, the portion of the toxin
5 comprises the receptor binding domain (*i.e.*, the C fragment of the toxin). The present invention is not limited by the nature of the non-toxin protein sequence employed. In a preferred embodiment, the non-toxin protein sequence comprises a poly-histidine tract. A number of alternative fusion tags or fusion partners are known to the art (*e.g.*, MBP, GST, protein A, etc.) and may be employed for the generation of fusion
10 proteins comprising vaccines. When a fusion partner (*i.e.*, the non-toxin protein sequence) is employed for the production of a recombinant *C. botulinum* toxin protein, the fusion partner may be removed from the recombinant *C. botulinum* toxin protein if desired (*i.e.*, prior to administration of the protein to a subject) using a variety of methods known to the art (*e.g.*, digestion of fusion proteins containing FactorXa or
15 thrombin recognition sites with the appropriate enzyme). A number of the pETHis vectors employed herein provide an N-terminal his-tag followed by a FactorXa cleavage site (see Example 28a); the botulinum C fragment sequences follow the FactorXa site and thus, FactorXa can be used to remove the his-tag from the botulinum fusion protein. In a preferred embodiment, the vaccine is substantially endotoxin-free.

20 The present invention is not limited by the method employed for the generation of vaccine comprising fusion proteins comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin. The fusion proteins may be produced by recombinant DNA means using either native or synthetic gene sequences expressed in a host cell. The present invention is not limited to the production of vaccines using
25 recombinant host cells; cell free *in vitro* transcription/translation systems may be employed for the expression of the nucleic acid constructs encoding the fusion proteins of the present invention. An example of such a cell-free system is the commercially

available TnT™ Coupled Reticulocyte Lysate System (Promega Corporation, Madison, WI). Alternatively, the fusion proteins of the present invention may be generated by synthetic means (*i.e.*, peptide synthesis).

5 The present invention further provides a method of generating antibody directed against a *Clostridium botulinum* toxin comprising: a) providing in any order: i) an antigen comprising a fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin, and ii) a host; and b) immunizing the host with the antigen so as to generate an antibody. In a preferred embodiment, the antigen
10 used to immunize the host also contains a fusion protein comprising a non-toxin protein sequence and at least a portion of *Clostridium botulinum* type A toxin. The present invention is not limited by the nature of the portion of the *Clostridium botulinum* toxin selected. In a preferred embodiment, the portion of the toxin comprises the receptor binding domain (*i.e.*, the C fragment of the toxin). The present
15 invention is not limited by the nature of the non-toxin protein sequence employed. In a preferred embodiment, the non-toxin protein sequence comprises a poly-histidine tract. A number of alternative fusion tags or fusion partners are known to the art (*e.g.*, MBP, GST, protein A, etc.) and may be employed for the generation of fusion proteins comprising vaccines. When a fusion partner (*i.e.*, the non-toxin protein
20 sequence) is employed for the production of a recombinant *C. botulinum* toxin protein, the fusion partner may be removed from the recombinant *C. botulinum* toxin protein if desired (*i.e.*, prior to administration of the protein to a subject) using a variety of methods known to the art (*e.g.*, digestion of fusion proteins containing FactorXa or thrombin recognition sites with the appropriate enzyme).

25 The present invention is not limited by the nature of the host employed for the production of the antibodies of the invention. In a preferred embodiment, the host is a mammal, preferably a human. The antibodies of the present invention may be

generated using non-mammalian hosts such as birds, preferably chickens. In a preferred embodiment the method of the present invention further comprised the step c) of collecting the antibodies from the host. In yet another embodiment, the method of the present invention further comprises the step d) of purifying the antibodies.

5 The present invention further provides antibodies raised according to the above methods.

10 The present invention further contemplates multivalent vaccines comprising at least two recombinant *C. botulinum* toxin proteins derived from the group consisting of *C. botulinum* serotypes A, B, C, D, E, F, and G. The invention contemplates bivalent, trivalent, quadravalent, pentavalent, heptavalent and septivalent vaccines comprising recombinant *C. botulinum* toxin proteins. Preferably the recombinant *C. botulinum* toxin protein comprises the receptor binding domain (*i.e.*, C fragment) of the toxin.

DESCRIPTION OF THE INVENTION

15 The present invention contemplates vaccinating humans and other animals with polypeptides derived from *C. botulinum* neurotoxins which are substantially endotoxin-free. These botulinal peptides are also useful for the production of antitoxin. Anti-botulinal toxin antitoxin is useful for the treatment of patients effected by or at risk of symptoms due to the action of *C. botulinum* toxins. The organisms, toxins and individual steps of the present invention are described separately below.

20 I. *Clostridium* Species, Clostridial Diseases And Associated Toxins

25 A preferred embodiment of the method of the present invention is directed toward obtaining antibodies against *Clostridium* species, their toxins, enzymes or other metabolic by-products, cell wall components, or synthetic or recombinant versions of any of these compounds. It is contemplated that these antibodies will be produced by immunization of humans or other animals. It is not intended that the present invention

be limited to any particular toxin or any species of organism. In one embodiment, toxins from all *Clostridium* species are contemplated as immunogens. Examples of these toxins include the neuraminidase toxin of *C. butyricum*, *C. sordellii* toxins HT and LT, toxins A, B, C, D, E, F, and G of *C. botulinum* and the numerous *C.*

perfringens toxins. In one preferred embodiment, toxins A, B, and E of *C. botulinum* are contemplated as immunogens. Table 2 above lists various *Clostridium* species, their toxins and some antigens associated with disease.

TABLE 2
Clostridial Toxins

Organism	Toxins and Disease-Associated Antigens
<i>C. botulinum</i>	A, B, C ₁ , C ₂ , D, E, F, G
<i>C. butyricum</i>	Neuraminidase
<i>C. difficile</i>	A, B, Enterotoxin (not A nor B), Motility Altering Factor, Low Molecular Weight Toxin, Others
<i>C. perfringens</i>	α , β , ϵ , ι , γ , δ , ν , θ , κ , λ , μ , υ
<i>C. sordellii</i> / <i>C. bifermentans</i>	HT, LT, α , β , γ
<i>C. novyi</i>	α , β , γ , δ , ϵ , ζ , ν , θ
<i>C. septicum</i>	α , β , γ , δ
<i>C. histolyticum</i>	α , β , γ , δ , ϵ plus additional enzymes
<i>C. chauvoei</i>	α , β , γ , δ

It is not intended that antibodies produced against one toxin will only be used against that toxin. It is contemplated that antibodies directed against one toxin (e.g., *C. perfringens* type A enterotoxin) may be used as an effective therapeutic against one or more toxin(s) produced by other members of the genus *Clostridium* or other toxin producing organisms (e.g., *Bacillus cereus*, *Staphylococcus aureus*, *Streptococcus mutans*, *Acinetobacter calcoaceticus*, *Pseudomonas aeruginosa*, other *Pseudomonas*

species, etc.). It is further contemplated that antibodies directed against the portion of the toxin which binds to mammalian membranes (e.g., *C. perfringens* enterotoxin A) can also be used against other organisms. It is contemplated that these membrane binding domains are produced synthetically and used as immunogens.

5 II. Obtaining Antibodies In Non-Mammals

A preferred embodiment of the method of the present invention for obtaining antibodies involves immunization. However, it is also contemplated that antibodies could be obtained from non-mammals without immunization. In the case where no immunization is contemplated, the present invention may use non-mammals with
10 preexisting antibodies to toxins as well as non-mammals that have antibodies to whole organisms by virtue of reactions with the administered antigen. An example of the latter involves immunization with synthetic peptides or recombinant proteins sharing epitopes with whole organism components.

In a preferred embodiment, the method of the present invention contemplates
15 immunizing non-mammals with bacterial toxin(s). It is not intended that the present invention be limited to any particular toxin. In one embodiment, toxin from all clostridial bacteria sources (see Table 2) are contemplated as immunogens. Examples of these toxins are *C. butyricum* neuraminidase toxin, toxins A, B, C, D, E, F, and G from *C. botulinum*, *C. perfringens* toxins α , β , ϵ , and i , and *C. sordellii* toxins HT and LT. In a preferred embodiment, *C. botulinum* toxins A, B, C, D, E, and F (or
20 fragments thereof) are contemplated as immunogens.

A particularly preferred embodiment involves the use of bacterial toxin protein or fragments of toxin proteins produced by molecular biological means (i.e., recombinant toxin proteins). In a preferred embodiment, the immunogen comprises
25 the receptor-binding domain (i.e., the ~50 kD carboxy-terminal portion of the heavy chain; also referred to as the C fragment) of *C. botulinum* serotype A neurotoxin produced by recombinant DNA technology. In another preferred embodiment, the immunogen comprises the receptor-binding domain of *C. botulinum* serotype B

neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype E neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype C1 neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype C2 neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype D neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype F neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype G neurotoxin produced by recombinant DNA technology. In a preferred embodiment, the recombinant botulinal toxin proteins are expressed as fusion proteins (e.g., as histidine-tagged proteins). In a still further preferred embodiment, the immunogen is a multivalent vaccine comprising the receptor-binding domain region of *C. botulinum* toxin from two or more toxins selected from the group consisting of type A, type B, type C (including C1 and C2), type D, type E, and type F toxin.

When immunization is used, the preferred non-mammal is from the class *Aves*. All birds are contemplated (e.g., duck, ostrich, emu, turkey, etc.). A preferred bird is a chicken. Importantly, chicken antibody does not fix mammalian complement. [See H.N. Benson *et al.*, J. Immunol. 87:616 (1961).] Thus, chicken antibody will normally not cause a complement-dependent reaction. [A.A. Benedict and K. Yamaga, "Immunoglobulins and Antibody Production in Avian Species," in *Comparative Immunology* (J.J. Marchaloni, ed.), pp. 335-375, Blackwell, Oxford (1966).] Thus, the preferred antitoxins of the present invention will not exhibit complement-related side effects observed with antitoxins known presently.

When birds are used, it is contemplated that the antibody will be obtained from either the bird serum or the egg. A preferred embodiment involves collection of the antibody from the egg. Laying hens transport immunoglobulin to the egg yolk ("IgY") in concentrations equal to or exceeding that found in serum. [See R. Patterson *et al.*, J. Immunol. 89:272 (1962); and S.B. Carroll and B.D. Stollar, J. Biol. Chem. 258:24 (1983).] In addition, the large volume of egg yolk produced vastly exceeds the volume of serum that can be safely obtained from the bird over any given time period. Finally, the antibody from eggs is purer and more homogeneous; there is far less non-immunoglobulin protein (as compared to serum) and only one class of immunoglobulin is transported to the yolk.

When considering immunization with toxins, one may consider modification of the toxins to reduce the toxicity. In this regard, it is not intended that the present invention be limited by immunization with modified toxin. Unmodified ("native") toxin is also contemplated as an immunogen.

It is also not intended that the present invention be limited by the type of modification -- if modification is used. The present invention contemplates all types of toxin modification, including chemical and heat treatment of the toxin. The preferred modification, however, is formaldehyde treatment.

It is not intended that the present invention be limited to a particular mode of immunization; the present invention contemplates all modes of immunization, including subcutaneous, intramuscular, intraperitoneal, and intravenous or intravascular injection, as well as *per os* administration of immunogen.

The present invention further contemplates immunization with or without adjuvant. (Adjuvant is defined as a substance known to increase the immune response to other antigens when administered with other antigens.) If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant -- or that the same adjuvant, once used, be used all the time. While the present invention contemplates all types of adjuvant, whether used separately or in combinations, the preferred use of adjuvant is the use of Complete Freund's Adjuvant followed sometime later with Incomplete Freund's Adjuvant. Another preferred use of adjuvant is the use

of Gerbu Adjuvant. The invention also contemplates the use of RIBI fowl adjuvant and Quil A adjuvant.

5 When immunization is used, the present invention contemplates a wide variety of immunization schedules. In one embodiment, a chicken is administered toxin(s) on day zero and subsequently receives toxin(s) in intervals thereafter. It is not intended that the present invention be limited by the particular intervals or doses. Similarly, it is not intended that the present invention be limited to any particular schedule for collecting antibody. The preferred collection time is sometime after day 100.

10 Where birds are used and collection of antibody is performed by collecting eggs, the eggs may be stored prior to processing for antibody. It is preferred that eggs be stored at 4°C for less than one year.

It is contemplated that chicken antibody produced in this manner can be buffer-extracted and used analytically. While unpurified, this preparation can serve as a reference for activity of the antibody prior to further manipulations (*e.g.*,
15 immunoaffinity purification).

III. Increasing The Effectiveness Of Antibodies

When purification is used, the present invention contemplates purifying to increase the effectiveness of both non-mammalian antitoxins and mammalian antitoxins. Specifically, the present invention contemplates increasing the percent of
20 toxin-reactive immunoglobulin. The preferred purification approach for avian antibody is polyethylene glycol (PEG) separation.

The present invention contemplates that avian antibody be initially purified using simple, inexpensive procedures. In one embodiment, chicken antibody from eggs is purified by extraction and precipitation with PEG. PEG purification exploits
25 the differential solubility of lipids (which are abundant in egg yolks) and yolk proteins in high concentrations of PEG 8000. [Polson *et al.*, Immunol. Comm. 9:495 (1980).] The technique is rapid, simple, and relatively inexpensive and yields an immunoglobulin fraction that is significantly purer in terms of contaminating non-immunoglobulin proteins than the comparable ammonium sulfate fractions of

mammalian sera and horse antibodies. The majority of the PEG is removed from the precipitated chicken immunoglobulin by treatment with ethanol. Indeed, PEG-purified antibody is sufficiently pure that the present invention contemplates the use of PEG-purified antitoxins in the passive immunization of intoxicated humans and animals.

5 IV. Treatment

The present invention contemplates antitoxin therapy for humans and other animals intoxicated by bacterial toxins. A preferred method of treatment is by intravenous administration of anti-boutlinal antitoxin; oral administration is also contemplated for other clostridial antitoxins.

10 A. Dosage Of Antitoxin

It was noted by way of background that a balance must be struck when administering currently available antitoxin which is usually produced in large animals such as horses; sufficient antitoxin must be administered to neutralize the toxin, but not so much antitoxin as to increase the risk of untoward side effects. These side
15 effects are caused by: i) patient sensitivity to foreign (e.g, horse) proteins; ii) anaphylactic or immunogenic properties of non-immunoglobulin proteins; iii) the complement fixing properties of mammalian antibodies; and/or iv) the overall burden of foreign protein administered. It is extremely difficult to strike this balance when, as noted above, the degree of intoxication (and hence the level of antitoxin therapy
20 needed) can only be approximated.

The present invention contemplates significantly reducing side effects so that this balance is more easily achieved. Treatment according to the present invention contemplates reducing side effects by using PEG-purified antitoxin from birds.

In one embodiment, the treatment of the present invention contemplates the use
25 of PEG-purified antitoxin from birds. The use of yolk-derived, PEG-purified antibody as antitoxin allows for the administration of: 1) non(mammalian)-complement-fixing, avian antibody; 2) a less heterogeneous mixture of non-immunoglobulin proteins; and 3) less total protein to deliver the equivalent weight of active antibody present in

currently available antitoxins. The non-mammalian source of the antitoxin makes it useful for treating patients who are sensitive to horse or other mammalian sera.

B. Delivery Of Antitoxin

Although it is not intended to limit the route of delivery, the present invention
5 contemplates a method for antitoxin treatment of bacterial intoxication in which
delivery of antitoxin is oral. In one embodiment, antitoxin is delivered in a solid form
(*e.g.*, tablets). In an alternative embodiment antitoxin is delivered in an aqueous
solution. When an aqueous solution is used, the solution has sufficient ionic strength
to solubilize antibody protein, yet is made palatable for oral administration. The
10 delivery solution may also be buffered (*e.g.*, carbonate buffer pH 9.5) which can
neutralize stomach acids and stabilize the antibodies when the antibodies are
administered orally. In one embodiment the delivery solution is an aqueous solution.
In another embodiment the delivery solution is a nutritional formula. Preferably, the
delivery solution is infant formula. Yet another embodiment contemplates the delivery
15 of lyophilized antibody encapsulated or microencapsulated inside acid-resistant
compounds.

Methods of applying enteric coatings to pharmaceutical compounds are well
known to the art [companies specializing in the coating of pharmaceutical compounds
are available; for example, The Coating Place (Verona, WI) and AAI (Wilmington,
20 NC)]. Enteric coatings which are resistant to gastric fluid and whose release (*i.e.*,
dissolution of the coating to release the pharmaceutical compound) is pH dependent
are commercially available [for example, the polymethacrylates Eudragit® L and
Eudragit® S (Röhm GmbH)]. Eudragit® S is soluble in intestinal fluid from pH 7.0;
this coating can be used to microencapsulate lyophilized antitoxin antibodies and the
25 particles are suspended in a solution having a pH above or below pH 7.0 for oral
administration. The microparticles will remain intact and undissolved until they
reached the intestines where the intestinal pH would cause them to dissolve thereby
releasing the antitoxin.

The invention contemplates a method of treatment which can be administered for treatment of acute intoxication. In one embodiment, antitoxin is administered orally in either a delivery solution or in tablet form, in therapeutic dosage, to a subject intoxicated by the bacterial toxin which served as immunogen for the antitoxin.

5 The invention also contemplates a method of treatment which can be administered prophylactically. In one embodiment, antitoxin is administered orally, in a delivery solution, in therapeutic dosage, to a subject, to prevent intoxication of the subject by the bacterial toxin which served as immunogen for the production of antitoxin. In another embodiment, antitoxin is administered orally in solid form such
10 as tablets or as microencapsulated particles. Microencapsulation of lyophilized antibody using compounds such as Eudragit® (Rohm GmbH) or polyethylene glycol , which dissolve at a wide range of pH units, allows the oral administration of solid antitoxin in a liquid form (*i.e.*, a suspension) to recipients unable to tolerate administration of tablets (*e.g.*, children or patients on feeding tubes). In one preferred
15 embodiment the subject is a child. In another embodiment, antibody raised against whole bacterial organism is administered orally to a subject, in a delivery solution, in therapeutic dosage.

V. Vaccines Against Clostridial Species

20 The invention contemplates the generation of mono- and multivalent vaccines for the protection of an animal (particularly humans) against several clostridial species. Of particular interest are vaccines which stimulate the production of a humoral immune response to *C. botulinum*, *C. tetani* and *C. difficile* in humans. The antigens comprising the vaccine preparation may be native or recombinantly produced toxin proteins from the clostridial species listed above. When toxin proteins are used as
25 immunogens they are generally modified to reduce the toxicity. This modification may be by chemical or genetic (*i.e.*, recombinant DNA technology) means. In general genetic detoxification (*i.e.*, the expression of nontoxic fragments in a host cell) is preferred as the expression of nontoxic fragments in a host cell precludes the presence

of intact, active toxin in the final preparation. However, when chemical modification is desired, the preferred toxin modification is formaldehyde treatment.

5 The invention contemplates that recombinant *C. botulinum* toxin proteins be used as antigens in mono- and multivalent vaccine preparations. Soluble, substantially endotoxin-free recombinant *C. botulinum* toxin proteins derived from serotypes A, B and E may be used individually (*i.e.*, as mono-valent vaccines) or in combination (*i.e.*, as a multi-valent vaccine). In addition, the recombinant *C. botulinum* toxin proteins derived from serotypes A, B and E may be used in conjunction with either recombinant or native toxins or toxoids from other serotypes of *C. botulinum*, *C. difficile* and *C.*
10 *tetani* as antigens for the preparation of these mono- and multivalent vaccines. It is contemplated that, due to the structural similarity of *C. botulinum* and *C. tetani* toxin proteins, a vaccine comprising *C. difficile* and *botulinum* toxin proteins (native or recombinant or a mixture thereof) be used to stimulate an immune response against *C. botulinum*, *C. tetani* and *C. difficile*.

15 The present invention further contemplates multi-valent vaccines comprising two or more botulinal toxin proteins selected from the group comprising recombinant *C. botulinum* toxin proteins derived from serotypes A, B, C (including C1 and C2), D, E, F and G.

20 The adverse consequences of exposure to botulinal toxin would be avoided by immunization of subjects at risk of exposure to the toxin with nontoxic preparations which confer immunity such as chemically or genetically detoxified toxin.

25 Vaccines which confer immunity against one or more of the toxin types A, B, E, F and G would be useful as a means of protecting humans from the deleterious effects of those *C. botulinum* toxins known to affect man. Indeed as the possibility exists that humans could be exposed to any of the seven serotypes of *C. botulinum* toxin (*e.g.*, during biological warfare or the production of toxin in a laboratory setting), multivalent vaccines capable of conferring immunity against toxin types A-G (including both C1 and C2 toxins) would be useful for the protection of humans.

Vaccines which confer immunity against one or more of the toxin types C, D and E would be useful for veterinary applications.

5 The botulinal neurotoxin is synthesized as a single polypeptide chain which is processed into a heavy (H; ~100 kD) and a light (L; ~50 kD) chain by cleavage with proteolytic enzymes; these two chains are held together via disulfide bonds in the active toxin (referred to as derivative toxin) [B.R. DasGupta and H. Sugiyama, Biochem. Biophys. Res. Commun. 48:108 (1972); reviewed in B.R. DasGupta, J. Physiol. 84:220 (1990), H. Sugiyama, Microbiol. Rev. 44:419 (1980) and C.L. Hatheway, Clin. Microbiol. Rev. 3:66 (1990)]. The heavy chain of the active toxin is
10 cleaved by trypsin to produce two fragments termed H_C (also referred to as H₁ or C) and H_N (also referred to as H₂ or B). The H_C fragment (~46 kD) comprises the carboxy end of the H chain. The H_N fragment (~49 kD) comprises the amino end and remains attached to the L chain (H_NL). Neither H_C or H_NL is toxic. H_C competes with whole derivative toxin for binding to synaptosomes and therefore H_C is said to contain the
15 receptor binding site. The H_C and H_N fragments of botulinal toxin are analogous to the fragments C and B of tetanus toxin which are produced by papain cleavage. The C fragment of tetanus toxin has been shown to be responsible for the binding of tetanus toxin to purified gangliosides and neuronal cells [Halpern and Loftus, J. Biol. Chem. 288:11188 (1993)].

20 Antisera raised against purified preparations of isolated botulinal H and L chains have been shown to protect mice against the lethal effects of the toxin; however, the effectiveness of the two antisera differ with the anti-H sera being more potent (H. Sugiyama, *supra*). While the different botulinal toxins show structural similarity to one another, the different serotypes are reported to be immunologically
25 distinct (*i.e.*, sera raised against one toxin type does not cross-react to a significant degree with other types). Thus, the generation of multivalent vaccines may require the use of more than one type of toxin.

C. botulinum toxin genes from all seven serotypes have been cloned and sequenced (Minton (1995), *supra*); in addition, partial amino acid sequence is available

for a number of *C. botulinum* toxins isolated from different strains within a given serotype. The *C. botulinum* toxins contain about 1250-1300 amino acid residues. On the DNA level, the overall degree of homology between *C. botulinum* serotypes A, B, C, D and E toxins averages between 50 and 60% identity with a greater degree of homology being found between H chain-encoding regions than between those encoding L chains [Whelan *et al.* (1992) Appl. Environ. Microbiol. 58:2345]. The degree of identity between *C. botulinum* toxins on the amino acid level reflects the level of DNA sequence homology. The most divergent area of DNA and amino acid sequence is found within the carboxy-terminal area of the various *C. botulinum* H chain genes. This portion of the toxin (*i.e.*, H_C or the C fragment) plays a major role in cell binding. As toxin from different serotypes is thought to bind to distinct cell receptor molecules, it is not surprising that the toxins diverge significantly over this region.

Within a given serotype, small variations in the primary amino acid sequence of the botulinum toxins isolated from different strains has been reported [Whelan *et al.* (1992), *supra* and Minton (1995), *supra*]. The present invention contemplates fusion proteins comprising portions of *C. botulinum* toxins from serotypes A-G including the variants found among different strains within a given serotype. The present invention provides oligonucleotide primers which may be used to amplify the C fragment or receptor-binding region of the toxin gene from various strains of *C. botulinum* serotype A, serotype B, serotype C (C1 and C2), serotype D, serotype E, serotype F and serotype G. A large number of different strains of *C. botulinum* serotype A, serotype B, serotype C, serotype D serotype E and serotype F are available from the American Type Culture Collection (ATCC; Rockville, MD). For example, the ATCC provides the following: Type A strains: 174 (ATCC 3502), 457 (ATCC 17862), and NCTC 7272 (ATCC 19397); Type B strains: 34 (ATCC 439), 62A (ATCC 7948), NCA 213 B (ATCC 7949), 13114 (ATCC 8083), 3137 (ATCC 17780), 1347 (ATCC 17841), 2017 (ATCC 17843), 2217 (ATCC 17844), 2254 (ATCC 17845) and VP 1731 (ATCC 25765); Type C strains: 2220 (ATCC 17782), 2239 (ATCC 17783), 2223 (ATCC 17784; a type C- β strain; C- β strains produce C2 toxin), 662 (ATCC 17849; a type C-

α strain; C- α strains produce mainly C1 toxin and a small amount of C2 toxin), 2021 (ATCC 17850; a type C- α strain) and VPI 3803 (ATCC 25766); Type D strains: ATCC 9633, 2023 (ATCC 17851), and VPI 5995 (ATCC 27517); Type E strains: ATCC 43181, 36208 (ATCC 9564), 2231 (ATCC 17786), 2229 (ATCC 17852), 2279 (ATCC 17854) and 2285 (ATCC 17855) and Type F strains: 202F (ATCC 23387), VPI 4404 (ATCC 25764), VPI 2382 (ATCC 27321) and Langeland (ATCC 35415). Type G strain, 113/30 (NCFB 3012) may be obtained from the National Collection of Food Bacteria (NCFB, AFRC Institute of Food Research, Reading, United Kingdom).

Purification methods have been reported for native toxin types A, B, C, D, E, and F [reviewed in G. Sakaguchi, *Pharmac. Ther.* 19:165 (1983)]. As the different botulinal toxins are structurally related, the invention contemplates the expression of any of the botulinal toxins (e.g., types A-G) as soluble recombinant fusion proteins.

In particular, methods for purification of the type A botulinum neurotoxin have been developed [L.J. Moberg and H. Sugiyama, *Appl. Environ. Microbiol.* 35:878 (1978)]. Immunization of hens with detoxified purified protein results in the generation of neutralizing antibodies [B.S. Thalley *et al.*, in *Botulinum and Tetanus Neurotoxins*, B.R. DasGupta, ed., Plenum Press, New York (1993), p. 467].

The currently available *C. botulinum* pentavalent vaccine comprising chemically inactivated (i.e., formaldehyde treated) type A, B, C, D and E toxins is not adequate. The efficacy is variable (in particular, only 78% of recipients produce protective levels of anti-type B antibodies following administration of the primary series) and immunization is painful (deep subcutaneous inoculation is required for administration), with adverse reactions being common (moderate to severe local reactions occur in approximately 6% of recipients upon initial injection; this number rises to approximately 11% of individuals who receive booster injections) [Informational Brochure for the Pentavalent (ABCDE) Botulinum Toxoid, Centers for Disease Control]. Preparation of this vaccine is dangerous as active toxin must be handled by laboratory workers.

In general, chemical detoxification of bacterial toxins using agents such as formaldehyde, glutaraldehyde or hydrogen peroxide is not optimal for the generation of vaccines or antitoxins. A delicate balance must be struck between too much and too little chemical modification. If the treatment is insufficient, the vaccine may retain residual toxicity. If the treatment is too excessive, the vaccine may lose potency due to destruction of native immunogenic determinants. Another major limitation of using botulinal toxoids for the generation of antitoxins or vaccines is the high production expense. For the above reasons, the development of methods for the production of nontoxic but immunogenic *C. botulinum* toxin proteins is desirable.

The *C. botulinum* and *C. tetanus* toxin proteins have similar structures [reviewed in E.J. Schantz and E.A. Johnson, Microbiol. Rev. 56:80 (1992)]. The carboxy-terminal 50 kD fragment of the tetanus toxin heavy chain (fragment C) is released by papain cleavage and has been shown to be non-toxic and immunogenic. Recombinant tetanus toxin fragment C has been developed as a candidate vaccine antigen [A.J. Makoff *et al.*, Bio/Technology 7:1043 (1989)]. Mice immunized with recombinant tetanus toxin fragment C were protected from challenge with lethal doses of tetanus toxin. No studies have demonstrated that the recombinant tetanus fragment C protein confers immunity against other botulinal toxins such as the *C. botulinum* toxins.

Recombinant tetanus fragment C has been expressed in *E. coli* [A.J. Makoff *et al.*, Bio/Technology, *supra* and Nucleic Acids Res. 17:10191 (1989); J.L. Halpern *et al.*, Infect. Immun. 58:1004 (1990)], yeast [M.A. Romanos *et al.*, Nucleic Acids Res. 19:1461 (1991)] and baculovirus [I.G. Charles *et al.*, Infect. Immun. 59:1627 (1991)]. Synthetic tetanus toxin genes had to be constructed to facilitate expression in yeast (M.A. Romanos *et al.*, *supra*) and *E. coli* [A.J. Makoff *et al.*, Nucleic Acids Res., *supra*], due to the high A-T content of the tetanus toxin gene sequences. High A-T content is a common feature of clostridial genes (M.R. Popoff *et al.*, Infect. Immun. 59:3673 (1991); H.F. LaPenotiere *et al.*, in *Botulinum and Tetanus Neurotoxins*, B.R. DasGupta, ed., Plenum Press, New York (1993), p. 463] which creates expression

difficulties in *E. coli* and yeast due primarily to altered codon usage frequency and fortuitous polyadenylation sites, respectively.

5 The C fragment of the *C. botulinum* type A neurotoxin heavy chain has been evaluated as a vaccine candidate. The *C. botulinum* type A neurotoxin gene has been cloned and sequenced [D.E. Thompson *et al.*, Eur. J. Biochem. 189:73 (1990)]. The C
10 fragment of the type A toxin was expressed as either a fusion protein comprising the botulinal C fragment fused with the maltose binding protein (MBP) or as a native protein [H.F. LaPenotiere *et al.*, (1993) *supra*, H.F. LaPenotiere *et al.*, Toxicon. 33:1383 (1995) and Middlebrook and Brown (1995), Curr. Top. Microbiol. Immunol. 195:89-122].
15 The plasmid construct encoding the native protein was reported to be unstable, while the fusion protein was expressed primarily in inclusion bodies as insoluble protein. Immunization of mice with crudely purified MBP fusion protein resulted in protection against IP challenge with 3 LD₅₀ doses of toxin [LaPenotiere *et al.*, (1993) and (1995), *supra*]. However, this recombinant *C. botulinum* type A toxin C fragment/MBP fusion protein is not a suitable immunogen for the production of
20 vaccines as it is expressed as an insoluble protein in *E. coli*. Furthermore, this recombinant *C. botulinum* type A toxin C fragment/MBP fusion protein was not shown to be substantially free of endotoxin contamination. Experience with recombinant *C. botulinum* type A toxin C fragment/MBP fusion proteins shows that the presence of
25 the MBP on the fusion protein greatly complicates the removal of endotoxin from preparations of the recombinant fusion protein (*see* Ex. 24, *infra*). Expression of a synthetic gene encoding *C. botulinum* type A toxin C fragment as a soluble protein excreted from insect cells has been reported [Middlebrook and Brown (1995), *supra*]; no details regarding the level of expression achieved or the presence of endotoxin or other pyrogens were provided. Like the insoluble protein expressed in *E. coli*, immunization with the recombinant protein produced in insect cells was reported to protect mice from challenge with *C. botulinum* toxin A.

Inclusion body protein must be solubilized prior to purification and/or administration to a host. The harsh treatment of inclusion body protein needed to

accomplish this solubilization may reduce the immunogenicity of the purified protein. Ideally, recombinant proteins to be used as vaccines are expressed as soluble proteins at high levels (*i.e.*, greater than or equal to about 0.75% of total cellular protein) in *E. coli* or other host cells (*e.g.*, yeast, insect cells, etc.). This facilitates the production and isolation of sufficient quantities of the immunogen in a highly purified form (*i.e.*, substantially free of endotoxin or other pyrogen contamination). The ability to express recombinant toxin proteins as soluble proteins in *E. coli* is advantageous due to the low cost of growth compared to insect or mammalian tissue culture cells.

The *C. botulinum* type B neurotoxin gene has been cloned and sequenced from two strains of *C. botulinum* type B [Whelan *et al.* (1992) Appl. Environ. Microbiol. 58:2345 (Danish strain) and Hutson *et al.* (1994) Curr. Microbiol. 28:101 (Eklund 17B strain)]. The nucleotide sequence of the toxin gene derived from the Eklund 17B strain (ATCC 25765) is available from the EMBL/GenBank sequence data banks under the accession number X71343; the nucleotide sequence of the coding region is listed in SEQ ID NO:39. The amino acid sequence of the *C. botulinum* type B neurotoxin derived from the strain Eklund 17B is listed in SEQ ID NO:40. The nucleotide sequence of the *C. botulinum* serotype B toxin gene derived from the Danish strain is listed in SEQ ID NO:41. The amino acid sequence of the *C. botulinum* type B neurotoxin derived from the Danish strain is listed in SEQ ID NO:42.

The *C. botulinum* type B neurotoxin gene is synthesized as a single polypeptide chain which is processed to form a dimer composed of a light and a heavy chain linked via disulfide bonds. The light chain is responsible for pharmacological activity (*i.e.*, inhibition of the release of acetylcholine at the neuromuscular junction). The N-terminal portion of the heavy chain is thought to mediate channel formation while the C-terminal portion mediates toxin binding; the type B neurotoxin has been reported to exist as a mixture of predominantly single chain with some double chain (Whelan *et al.*, *supra*). The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H_C domain. The present invention reports for the first time, the

expression of the C fragment of *C. botulinum* type B toxin in heterologous hosts (e.g., *E. coli*).

The *C. botulinum* type E neurotoxin gene has been cloned and sequenced from a number of different strains [Poulet *et al.* (1992) Biochem. Biophys. Res. Commun. 183:107; Whelan *et al.* (1992) Eur. J. Biochem. 204:657; and Fujii *et al.* (1993) J. Gen. Microbiol. 139:79]. The nucleotide sequence of the type E toxin gene is available from the EMBL sequence data bank under accession numbers X62089 (strain Beluga) and X62683 (strain NCTC 11219); the nucleotide sequence of the coding region (strain Beluga) is listed in SEQ ID NO:45. The amino acid sequence of the *C. botulinum* type E neurotoxin derived from strain Beluga is listed in SEQ ID NO:46. The type E neurotoxin gene is synthesized as a single polypeptide chain which may be converted to a double-chain form (*i.e.*, a heavy chain and a light chain) by cleavage with trypsin; unlike the type A neurotoxin, the type E neurotoxin exists essentially only in the single-chain form. The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H_C domain. The present invention reports for the first time, the expression of the C fragment of *C. botulinum* type E toxin in heterologous hosts (e.g., *E. coli*).

The *C. botulinum* type C1, D, F and G neurotoxin genes have been cloned and sequenced. The nucleotide and amino acid sequences of these genes and toxins are provided herein. The invention provides methods for the expression of the C fragment from each of these toxin genes in heterologous hosts and the purification of the resulting recombinant proteins.

The subject invention provides methods which allow the production of soluble *C. botulinum* toxin proteins in economical host cells (e.g., *E. coli*). In addition the subject invention provides methods which allow the production of soluble botulinal toxin proteins in yeast and insect cells. Further, methods for the isolation of purified soluble *C. botulinum* toxin proteins which are suitable for immunization of humans and other animals are provided. These soluble, purified preparations of *C. botulinum*

toxin proteins provide the basis for improved vaccine preparations and facilitate the production of antitoxin.

When recombinant clostridial toxin proteins produced in gram-negative bacteria (e.g., *E. coli*) are used as vaccines, they are purified to remove endotoxin prior to administration to a host animal. In order to vaccinate a host, an immunogenically-effective amount of purified substantially endotoxin-free recombinant clostridial toxin protein is administered in any of a number of physiologically acceptable carriers known to the art. When administered for the purpose of vaccination, the purified substantially endotoxin-free recombinant clostridial toxin protein may be used alone or in conjunction with known adjuvants, including potassium alum, aluminum phosphate, aluminum hydroxide, Gerbu adjuvant (GmDP; C.C. Biotech Corp.), RIBI adjuvant (MPL; RIBI Immunochemical Research, Inc.), QS21 (Cambridge Biotech). The alum and aluminum-based adjuvants are particularly preferred when vaccines are to be administered to humans; however, any adjuvant approved for use in humans may be employed. The route of immunization may be nasal, oral, intramuscular, intraperitoneal or subcutaneous.

The invention contemplates the use of soluble, substantially endotoxin-free preparations of fusion proteins comprising the C fragment of the *C. botulinum* type A, B, C, D, E, F, and G toxin as vaccines. In one embodiment, the vaccine comprises the C fragment of either the *C. botulinum* type A, B, C, D, E, F, or G toxin and a poly-histidine tract (also called a histidine tag). In a particularly preferred embodiment, a fusion protein comprising the histidine tagged C fragment is expressed using the pET series of expression vectors (Novagen). The pET expression system utilizes a vector containing the T7 promoter which encodes the fusion protein and a host cell which can be induced to express the T7 DNA polymerase (i.e., a DE3 host strain). The production of C fragment fusion proteins containing a histidine tract is not limited to the use of a particular expression vector and host strain. Several commercially available expression vectors and host strains can be used to express the C fragment protein sequences as a fusion protein containing a histidine tract (For example, the pQE series (pQE-8, 12, 16, 17, 18, 30, 31, 32, 40, 41, 42, 50, 51, 52, 60 and 70) of

expression vectors (Qiagen) which are used with the host strains M15[pREP4] (Qiagen) and SG13009[pREP4] (Qiagen) can be used to express fusion proteins containing six histidine residues at the amino-terminus of the fusion protein).

Furthermore a number of commercially available expression vectors which provide a histidine tract also provide a protease cleavage site between the histidine tract and the protein of interest (*e.g.*, botulinal toxin sequences). Cleavage of the resulting fusion protein with the appropriate protease will remove the histidine tag from the protein of interest (*e.g.*, botulinal toxin sequences) (see Example 28a, *infra*). Removal of the histidine tag may be desirable prior to administration of the recombinant botulinal toxin protein to a subject (*e.g.*, a human).

VI. Detection Of Toxin

The invention contemplates detecting bacterial toxin in a sample. The term "sample" in the present specification and claims is used in its broadest sense. On the one hand it is meant to include a specimen or culture. On the other hand, it is meant to include both biological and environmental samples.

Biological samples may be animal, including human, fluid, solid (*e.g.*, stool) or tissue; liquid and solid food products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The invention contemplates detecting bacterial toxin by a competitive immunoassay method that utilizes recombinant toxin A and toxin B proteins, antibodies raised against recombinant bacterial toxin proteins. A fixed amount of the recombinant toxin proteins are immobilized to a solid support (*e.g.*, a microtiter plate) followed by the addition of a biological sample suspected of containing a bacterial toxin. The biological sample is first mixed with affinity-purified or PEG fractionated

antibodies directed against the recombinant toxin protein. A reporter reagent is then added which is capable of detecting the presence of antibody bound to the immobilized toxin protein. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. If toxin is present in the sample, this toxin will compete with the immobilized recombinant toxin protein for binding to the anti-recombinant antibody thereby reducing the signal obtained following the addition of the reporter reagent. A control is employed where the antibody is not mixed with the sample. This gives the highest (or reference) signal.

The invention also contemplates detecting bacterial toxin by a "sandwich" immunoassay method that utilizes antibodies directed against recombinant bacterial toxin proteins. Affinity-purified antibodies directed against recombinant bacterial toxin proteins are immobilized to a solid support (*e.g.*, microtiter plates). Biological samples suspected of containing bacterial toxins are then added followed by a washing step to remove substantially all unbound antitoxin. The biological sample is next exposed to the reporter substance, which binds to antitoxin and is then washed free of substantially all unbound reporter substance. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. Identification of the reporter substance in the biological tissue indicates the presence of the bacterial toxin.

It is also contemplated that bacterial toxin be detected by pouring liquids (*e.g.*, soups and other fluid foods and feeds including nutritional supplements for humans and other animals) over immobilized antibody which is directed against the bacterial toxin. It is contemplated that the immobilized antibody will be present in or on such supports as cartridges, columns, beads, or any other solid support medium. In one embodiment, following the exposure of the liquid to the immobilized antibody, unbound toxin is substantially removed by washing. The exposure of the liquid is then exposed to a reporter substance which detects the presence of bound toxin. In a preferred embodiment the reporter substance is an enzyme, fluorescent dye, or radioactive compound attached to an antibody which is directed against the toxin (*i.e.*,

in a "sandwich" immunoassay). It is also contemplated that the detection system will be developed as necessary (e.g., the addition of enzyme substrate in enzyme systems; observation using fluorescent light for fluorescent dye systems; and quantitation of radioactivity for radioactive systems).

5 **EXPERIMENTAL**

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

10 In the disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); rpm (revolutions per minute); BBS-Tween (borate buffered saline containing Tween); BSA (bovine serum albumin); ELISA (enzyme-linked immunosorbent assay); CFA (complete Freund's adjuvant); IFA (incomplete Freund's adjuvant); IgG (immunoglobulin G); IgY (immunoglobulin Y); IM (intramuscular); IP (intraperitoneal); IV (intravenous or intravascular); SC (subcutaneous); H₂O (water);
15 HCl (hydrochloric acid); LD₁₀₀ (lethal dose for 100% of experimental animals); aa (amino acid); HPLC (high performance liquid chromatography); kD (kilodaltons); gm (grams); µg (micrograms); mg (milligrams); ng (nanograms); µl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm (micrometer); M (molar); mM (millimolar); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours);
20 MgCl₂ (magnesium chloride); NaCl (sodium chloride); Na₂CO₃ (sodium carbonate); OD₂₈₀ (optical density at 280 nm); OD₆₀₀ (optical density at 600 nm); PAGE (polyacrylamide gel electrophoresis); PBS [phosphate buffered saline (150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2)]; PEG (polyethylene glycol); PMSF (phenylmethylsulfonyl fluoride); SDS (sodium dodecyl sulfate); Tris
25 (tris(hydroxymethyl)aminomethane); Ensure® (Ensure®, Ross Laboratories, Columbus OH); Enfamil® (Enfamil®, Mead Johnson); w/v (weight to volume); v/v (volume to volume); Amicon (Amicon, Inc., Beverly, MA); Amresco (Amresco, Inc., Solon, OH); ATCC (American Type Culture Collection, Rockville, MD); BBL (Baltimore Biologics Laboratory, (a division of Becton Dickinson), Cockeysville, MD); Becton Dickinson

(Becton Dickinson Labware, Lincoln Park, NJ); BioRad (BioRad, Richmond, CA); Biotech (C-C Biotech Corp., Poway, CA); Charles River (Charles River Laboratories, Wilmington, MA); Cocalico (Cocalico Biologicals Inc., Reamstown, PA); CytRx (CytRx Corp., Norcross, GA); Falcon (e.g. Baxter Healthcare Corp., McGaw Park, IL and Becton Dickinson); FDA (Federal Food and Drug Administration); Fisher Biotech (Fisher Biotech, Springfield, NJ); GIBCO (Grand Island Biologic Company/BRL, Grand Island, NY); Gibco-BRL (Life Technologies, Inc., Gaithersburg, MD); Harlan Sprague Dawley (Harlan Sprague Dawley, Inc., Madison, WI); Mallinckrodt (a division of Baxter Healthcare Corp., McGaw Park, IL); Millipore (Millipore Corp., Marlborough, MA); New England Biolabs (New England Biolabs, Inc., Beverly, MA); Novagen (Novagen, Inc., Madison, WI); Pharmacia (Pharmacia, Inc., Piscataway, NJ); Qiagen (Qiagen, Chatsworth, CA); Sasco (Sasco, Omaha, NE); Showdex (Showa Denko America, Inc., New York, NY); Sigma (Sigma Chemical Co., St. Louis, MO); Sterogene (Sterogene, Inc., Arcadia, CA); Tech Lab (Tech Lab, Inc., Blacksburg, VA); and Vaxcell (Vaxcell, Inc., a subsidiary of CytRX Corp., Norcross, GA).

When a recombinant protein is described in the specification it is referred to in a short-hand manner by the amino acids in the toxin sequence present in the recombinant protein rounded to the nearest 10. For example, the recombinant protein pMB1850-2360 contains amino acids 1852 through 2362 of the *C. difficile* toxin B protein. The specification gives detailed construction details for all recombinant proteins such that one skilled in the art will know precisely which amino acids are present in a given recombinant protein.

EXAMPLE 1

Production Of High-Titer Antibodies To *Clostridium difficile* Organisms In A Hen

Antibodies to certain pathogenic organisms have been shown to be effective in treating diseases caused by those organisms. It has not been shown whether antibodies can be raised, against *Clostridium difficile*, which would be effective in treating

infection by this organism. Accordingly, *C. difficile* was tested as immunogen for production of hen antibodies.

To determine the best course for raising high-titer egg antibodies against whole *C. difficile* organisms, different immunizing strains and different immunizing concentrations were examined. The example involved (a) preparation of the bacterial immunogen, (b) immunization, (c) purification of anti-bacterial chicken antibodies, and (d) detection of anti-bacterial antibodies in the purified IgY preparations.

a) Preparation Of Bacterial Immunogen

C. difficile strains 43594 (serogroup A) and 43596 (serogroup C) were originally obtained from the ATCC. These two strains were selected because they represent two of the most commonly-occurring serogroups isolated from patients with antibiotic-associated pseudomembranous colitis. [Delmee *et al.*, J. Clin. Microbiol., 28(10):2210 (1990).] Additionally, both of these strains have been previously characterized with respect to their virulence in the Syrian hamster model for *C. difficile* infection. [Delmee *et al.*, J. Med Microbiol., 33:85 (1990).]

The bacterial strains were separately cultured on brain heart infusion agar for 48 hours at 37°C in a Gas Pack 100 Jar (BBL, Cockeysville, MD) equipped with a Gas Pack Plus anaerobic envelope (BBL). Forty-eight hour cultures were used because they produce better growth and the organisms have been found to be more cross-reactive with respect to their surface antigen presentation. The greater the degree of cross-reactivity of our IgY preparations, the better the probability of a broad range of activity against different strains/serogroups. [Toma *et al.*, J. Clin. Microbiol., 26(3):426 (1988).]

The resulting organisms were removed from the agar surface using a sterile dacron-tip swab, and were suspended in a solution containing 0.4% formaldehyde in PBS, pH 7.2. This concentration of formaldehyde has been reported as producing good results for the purpose of preparing whole-organism immunogen suspensions for the generation of polyclonal anti-*C. difficile* antisera in rabbits. [Delmee *et al.*, J. Clin.

Microbiol., 21:323 (1985); Davies *et al.*, Microbial Path., 9:141 (1990).] In this manner, two separate bacterial suspensions were prepared, one for each strain. The two suspensions were then incubated at 4°C for 1 hour. Following this period of formalin-treatment, the suspensions were centrifuged at $4,200 \times g$ for 20 min., and the resulting pellets were washed twice in normal saline. The washed pellets, which contained formalin-treated whole organisms, were resuspended in fresh normal saline such that the visual turbidity of each suspension corresponded to a #7 McFarland standard. [M.A.C. Edelstein, "Processing Clinical Specimens for Anaerobic Bacteria: Isolation and Identification Procedures," in S.M. Finegold *et al* (eds.), *Bailey and Scott's Diagnostic Microbiology*, pp. 477-507, C.V. Mosby Co., (1990). The preparation of McFarland nephelometer standards and the corresponding approximate number of organisms for each tube are described in detail at pp. 172-173 of this volume.] Each of the two #7 suspensions was then split into two separate volumes. One volume of each suspension was volumetrically adjusted, by the addition of saline, to correspond to the visual turbidity of a #1 McFarland standard. [*Id.*] The #1 suspensions contained approximately 3×10^8 organisms/ml, and the #7 suspensions contained approximately 2×10^9 organisms/ml. [*Id.*] The four resulting concentration-adjusted suspensions of formalin-treated *C. difficile* organisms were considered to be "bacterial immunogen suspensions." These suspensions were used immediately after preparation for the initial immunization. [See section (b).]

The formalin-treatment procedure did not result in 100% non-viable bacteria in the immunogen suspensions. In order to increase the level of killing, the formalin concentration and length of treatment were both increased for subsequent immunogen preparations, as described below in Table 3. (Although viability was decreased with the stronger formalin treatment, 100% inviability of the bacterial immunogen suspensions was not reached.) Also, in subsequent immunogen preparations, the formalin solutions were prepared in normal saline instead of PBS. At day 49, the day of the fifth immunization, the excess volumes of the four previous bacterial

immunogen suspensions were stored frozen at -70°C for use during all subsequent immunizations.

b) Immunization

For the initial immunization, 1.0 ml volumes of each of the four bacterial immunogen suspensions described above were separately emulsified in 1.2 ml volumes of CFA (GIBCO). For each of the four emulsified immunogen suspensions, two four-month old White Leghorn hens (pre-laying) were immunized. (It is not necessary to use pre-laying hens; actively-laying hens can also be utilized.) Each hen received a total volume of approximately 1.0 ml of a single emulsified immunogen suspension via four injections (two subcutaneous and two intramuscular) of approximately 250 µl per site. In this manner, a total of four different immunization combinations, using two hens per combination, were initiated for the purpose of evaluating both the effect of immunizing concentration on egg yolk antibody (IgY) production, and interstrain cross-reactivity of IgY raised against heterologous strains. The four immunization groups are summarized in Table 3.

TABLE 3

Immunization Groups

Group Designation	Immunizing Strain	Approximate Immunizing Dose
CD 43594, #1	<i>C. difficile</i> strain 43594	1.5×10^8 organisms/hen
CD 43594, #7	" "	1.0×10^9 organisms/hen
CD 43596, #1	<i>C. difficile</i> strain 43596	1.5×10^8 organisms/hen
CD 43596, #7	" "	1.0×10^9 organisms/hen

The time point for the first series of immunizations was designated as "day zero." All subsequent immunizations were performed as described above except that the bacterial immunogen suspensions were emulsified using IFA (GIBCO) instead of CFA, and for the later time point immunization, the stored frozen suspensions were

used instead of freshly-prepared suspensions. The immunization schedule used is listed in Table 4.

TABLE 4

Immunization Schedule

Day Of Immunization	Formalin-Treatment	Immunogen Preparation Used
0	1%, 1 hr.	freshly-prepared
14	1%, overnight	" "
21	1%, overnight	" "
35	1%, 48 hrs.	" "
49	1%, 72 hrs.	" "
70	" "	stored frozen
85	" "	" "
105	" "	" "

c) Purification Of Anti-Bacterial Chicken Antibodies

Groups of four eggs were collected per immunization group between days 80 and 84 post-initial immunization, and chicken immunoglobulin (IgY) was extracted according to a modification of the procedure of A. Polson *et al.*, Immunol. Comm., 9:495 (1980). A gentle stream of distilled water from a squirt bottle was used to separate the yolks from the whites, and the yolks were broken by dropping them through a funnel into a graduated cylinder. The four individual yolks were pooled for each group. The pooled, broken yolks were blended with 4 volumes of egg extraction buffer to improve antibody yield (egg extraction buffer is 0.01 M sodium phosphate, 0.1 M NaCl, pH 7.5, containing 0.005% thimerosal), and PEG 8000 (Amresco) was added to a concentration of 3.5%. When all the PEG dissolved, the protein precipitates that formed were pelleted by centrifugation at $13,000 \times g$ for 10 minutes. The supernatants were decanted and filtered through cheesecloth to remove the lipid layer, and the PEG was added to the supernatants to a final concentration of 12% (the supernatants were assumed to contain 3.5% PEG). After a second centrifugation, the supernatants were discarded and the pellets were centrifuged a final time to extrude the

remaining PEG. These crude IgY pellets were then dissolved in the original yolk volume of egg extraction buffer and stored at 4°C. As an additional control, a preimmune IgY solution was prepared as described above, using eggs collected from unimmunized hens.

5 **d) Detection Of Anti-Bacterial Antibodies In The
Purified IgY Preparations**

In order to evaluate the relative levels of specific anti-*C. difficile* activity in the IgY preparations described above, a modified version of the whole-organism ELISA procedure of N.V. Padhye *et al.*, J. Clin. Microbiol. 29:99-103 (1990) was used.

10 Frozen organisms of both *C. difficile* strains described above were thawed and diluted to a concentration of approximately 1×10^7 organisms/ml using PBS, pH 7.2. In this way, two separate coating suspensions were prepared, one for each immunizing strain. Into the wells of 96-well microtiter plates (Falcon, Pro-Bind Assay Plates) were placed 100 µl volumes of the coating suspensions. In this manner, each plate well received a
15 total of approximately 1×10^6 organisms of one strain or the other. The plates were then incubated at 4°C overnight. The next morning, the coating suspensions were decanted, and all wells were washed three times using PBS. In order to block non-specific binding sites, 100 µl of 0.5% BSA (Sigma) in PBS was then added to each well, and the plates were incubated for 2 hours at room temperature. The blocking
20 solution was decanted, and 100 µl volumes of the IgY preparations described above were initially diluted 1:500 with a solution of 0.1% BSA in PBS, and then serially diluted in 1:5 steps. The following dilutions were placed in the wells: 1:500, 1:2,500, 1:62,500, 1:312,500, and 1:1,562,500. The plates were again incubated for 2 hours at room temperature. Following this incubation, the IgY-containing solutions were
25 decanted, and the wells were washed three times using BBS-Tween (0.1 M boric acid, 0.025 M sodium borate, 1.0 M NaCl, 0.1% Tween-20), followed by two washes using PBS-Tween (0.1% Tween-20), and finally, two washes using PBS only. To each well, 100 µl of a 1:750 dilution of rabbit anti-chicken IgG (whole-molecule)-alkaline phosphatase conjugate (Sigma) (diluted in 0.1% BSA in PBS) was added. The plates

were again incubated for 2 hours at room temperature. The conjugate solutions were decanted and the plates were washed as described above, substituting 50 mM Na₂CO₃, pH 9.5 for the PBS in the final wash. The plates were developed by the addition of 100 µl of a solution containing 1 mg/ml para-nitrophenyl phosphate (Sigma) dissolved in 50 mM Na₂CO₃, 10 mM MgCl₂, pH 9.5 to each well, and incubating the plates at room temperature in the dark for 45 minutes. The absorbance of each well was measured at 410 nm using a Dynatech MR 700 plate reader. In this manner, each of the four IgY preparations described above was tested for reactivity against both of the immunizing *C. difficile* strains; strain-specific, as well as cross-reactive activity was determined.

Table 5 shows the results of the whole-organism ELISA. All four IgY preparations demonstrated significant levels of activity, to a dilution of 1:62,500 or greater against both of the immunizing organism strains. Therefore, antibodies raised against one strain were highly cross-reactive with the other strain, and vice versa. The immunizing concentration of organisms did not have a significant effect on organism-specific IgY production, as both concentrations produced approximately equivalent responses. Therefore, the lower immunizing concentration of approximately 1.5×10^8 organisms/hen is the preferred immunizing concentration of the two tested. The preimmune IgY preparation appeared to possess relatively low levels of *C. difficile*-reactive activity to a dilution of 1:500, probably due to prior exposure of the animals to environmental clostridia.

An initial whole-organism ELISA was performed using IgY preparations made from single CD 43594, #1 and CD 43596, #1 eggs collected around day 50 (data not shown). Specific titers were found to be 5 to 10-fold lower than those reported in Table 5. These results demonstrate that it is possible to begin immunizing hens prior to the time that they begin to lay eggs, and to obtain high titer specific IgY from the first eggs that are laid. In other words, it is not necessary to wait for the hens to begin laying before the immunization schedule is started.

TABLE 5

Results Of The Anti-*C. difficile* Whole-Organism ELISA

IgY Preparation	Dilution Of IgY Prep	43594-Coated Wells	43596-Coated Wells
CD 43594, #1	1:500	1.746	1.801
	1:2,500	1.092	1.670
	1:12,500	0.202	0.812
	1:62,500	0.136	0.179
	1:312,500	0.012	0.080
	1:1,562,500	0.002	0.020
CD 43594, #7	1:500	1.780	1.771
	1:2,500	1.025	1.078
	1:12,500	0.188	0.382
	1:62,500	0.052	0.132
	1:312,500	0.022	0.043
	1:1,562,500	0.005	0.024
CD 43596, #1	1:500	1.526	1.790
	1:2,500	0.832	1.477
	1:12,500	0.247	0.452
	1:62,500	0.050	0.242
	1:312,500	0.010	0.067
	1:1,562,500	0.000	0.036
CD 43596, #7	1:500	1.702	1.505
	1:2,500	0.706	0.866
	1:12,500	0.250	0.282
	1:62,500	0.039	0.078
	1:312,500	0.002	0.017
	1:1,562,500	0.000	0.010
Preimmune IgY	1:500	0.142	0.309
	1:2,500	0.032	0.077
	1:12,500	0.006	0.024
	1:62,500	0.002	0.012
	1:312,500	0.004	0.010
	1:1,562,500	0.002	0.014

EXAMPLE 2

Treatment Of *C. difficile* Infection With Anti-*C. difficile* Antibody

In order to determine whether the immune IgY antibodies raised against whole *C. difficile* organisms were capable of inhibiting the infection of hamsters by *C. difficile*, hamsters infected by these bacteria were utilized. [Lyerly *et al.*, Infect. Immun., 59:2215-2218 (1991).] This example involved: (a) determination of the

lethal dose of *C. difficile* organisms; and (b) treatment of infected animals with immune antibody or control antibody in nutritional solution.

a) **Determination Of The Lethal Dose Of *C. difficile* Organisms**

5 Determination of the lethal dose of *C. difficile* organisms was carried out according to the model described by D.M. Lyster *et al.*, Infect. Immun., 59:2215-2218 (1991). *C. difficile* strain ATCC 43596 (serogroup C, ATCC) was plated on BHI agar and grown anaerobically (BBL Gas Pak 100 system) at 37°C for 42 hours. Organisms were removed from the agar surface using a sterile dacron-tip swab and suspended in
10 sterile 0.9% NaCl solution to a density of 10^8 organisms/ml.

In order to determine the lethal dose of *C. difficile* in the presence of control antibody and nutritional formula, non-immune eggs were obtained from unimmunized hens and a 12% PEG preparation made as described in Example 1(c). This preparation was redissolved in one fourth the original yolk volume of vanilla flavor
15 Ensure®.

Starting on day one, groups of female Golden Syrian hamsters (Harlan Sprague Dawley), 8-9 weeks old and weighing approximately 100 gm, were orally administered 1 ml of the preimmune/Ensure® formula at time zero, 2 hours, 6 hours, and 10 hours. At 1 hour, animals were orally administered 3.0 mg clindamycin HCl (Sigma) in 1 ml
20 of water. This drug predisposes hamsters to *C. difficile* infection by altering the normal intestinal flora. On day two, the animals were given 1 ml of the preimmune IgY/Ensure® formula at time zero, 2 hours, 6 hours, and 10 hours. At 1 hour on day two, different groups of animals were inoculated orally with saline (control), or 10^2 , 10^4 , 10^6 , or 10^8 *C. difficile* organisms in 1 ml of saline. From days 3-12, animals were
25 given 1 ml of the preimmune IgY/Ensure® formula three times daily and observed for the onset of diarrhea and death. Each animal was housed in an individual cage and was offered food and water *ad libitum*.

Administration of 10^6 - 10^8 organisms resulted in death in 3-4 days while the lower doses of 10^2 - 10^4 organisms caused death in 5 days. Cecal swabs taken from dead animals indicated the presence of *C. difficile*. Given the effectiveness of the 10^2 dose, this number of organisms was chosen for the following experiment to see if hyperimmune anti-*C. difficile* antibody could block infection.

b) **Treatment Of Infected Animals With Immune Antibody Or Control Antibody In Nutritional Formula**

The experiment in (a) was repeated using three groups of seven hamsters each. Group A received no clindamycin or *C. difficile* and was the survival control. Group B received clindamycin, 10^2 *C. difficile* organisms and preimmune IgY on the same schedule as the animals in (a) above. Group C received clindamycin, 10^2 *C. difficile* organisms, and hyperimmune anti-*C. difficile* IgY on the same schedule as Group B. The anti-*C. difficile* IgY was prepared as described in Example 1 except that the 12% PEG preparation was dissolved in one fourth the original yolk volume of Ensure®.

All animals were observed for the onset of diarrhea or other disease symptoms and death. Each animal was housed in an individual cage and was offered food and water *ad libitum*. The results are shown in Table 6.

TABLE 6

The Effect Of Oral Feeding Of Hyperimmune IgY Antibody on *C. difficile* Infection

Animal Group	Time To Diarrhea*	Time To Death*
A pre-immune IgY only	no diarrhea	no deaths
B Clindamycin, <i>C. difficile</i> , preimmune IgY	30 hrs.	49 hrs.
C Clindamycin, <i>C. difficile</i> , immune IgY	33 hrs.	56 hrs.

* Mean of seven animals.

Hamsters in the control group A did not develop diarrhea and remained healthy during the experimental period. Hamsters in groups B and C developed diarrheal disease. Anti-*C. difficile* IgY did not protect the animals from diarrhea or death, all

5 animals succumbed in the same time interval as the animals treated with preimmune IgY. Thus, while immunization with whole organisms apparently can improve sub-lethal symptoms with particular bacteria (see U.S. Patent No. 5,080,895 to H. Tokoro), such an approach does not prove to be productive to protect against the lethal effects of *C. difficile*.

EXAMPLE 3

Production of *C. botulinum* Type A Antitoxin in Hens

10 In order to determine whether antibodies could be raised against the toxin produced by clostridial pathogens, which would be effective in treating clostridial diseases, antitoxin to *C. botulinum* type A toxin was produced. This example involves: (a) toxin modification; (b) immunization; (c) antitoxin collection; (d) antigenicity assessment; and (e) assay of antitoxin titer.

a) Toxin Modification

15 *C. botulinum* type A toxoid was obtained from B. R. DasGupta. From this, the active type A neurotoxin (M.W. approximately 150 kD) was purified to greater than 99% purity, according to published methods. [B.R. DasGupta & V. Sathyamoorthy, Toxicon, 22:415 (1984).] The neurotoxin was detoxified with formaldehyde according to published methods. [B.R. Singh & B.R. DasGupta, Toxicon, 27:403 (1989).]

b) Immunization

20 *C. botulinum* toxoid for immunization was dissolved in PBS (1 mg/ml) and was emulsified with an approximately equal volume of CFA (GIBCO) for initial immunization or IFA for booster immunization. On day zero, two white leghorn hens, obtained from local breeders, were each injected at multiple sites (intramuscular and subcutaneous) with 1 ml inactivated toxoid emulsified in 1 ml CFA. Subsequent
25 booster immunizations were made according to the following schedule for day of

injection and toxoid amount: days 14 and 21 - 0.5 mg; day 171 - 0.75 mg; days 394, 401, 409 - 0.25 mg. One hen received an additional booster of 0.150 mg on day 544.

c) **Antitoxin Collection**

5 Total yolk immunoglobulin (IgY) was extracted as described in Example 1(c) and the IgY pellet was dissolved in the original yolk volume of PBS with thimerosal.

d) **Antigenicity Assessment**

Eggs were collected from day 409 through day 423 to assess whether the toxoid was sufficiently immunogenic to raise antibody. Eggs from the two hens were pooled and antibody was collected as described in the standard PEG protocol.

10 [Example 1(c).] Antigenicity of the botulinal toxin was assessed on Western blots. The 150 kD detoxified type A neurotoxin and unmodified, toxic, 300 kD botulinal type A complex (toxin used for intragastric route administration for animal gut neutralization experiments; see Example 6) were separated on a SDS-polyacrylamide reducing gel. The Western blot technique was performed according to the method of
15 Towbin. [H. Towbin *et al.*, Proc. Natl. Acad. Sci. USA, 76:4350 (1979).] Ten µg samples of *C. botulinum* complex and toxoid were dissolved in SDS reducing sample buffer (1% SDS, 0.5% 2-mercaptoethanol, 50 mM Tris, pH 6.8, 10% glycerol, 0.025% w/v bromphenol blue, 10% β-mercaptoethanol), heated at 95°C for 10 min and separated on a 1 mm thick 5% SDS-polyacrylamide gel. [K. Weber and M.
20 Osborn, "Proteins and Sodium Dodecyl Sulfate: Molecular Weight Determination on Polyacrylamide Gels and Related Procedures," in *The Proteins*, 3d Edition (H. Neurath & R.L. Hill, eds), pp. 179-223, (Academic Press, NY, 1975).] Part of the gel was cut off and the proteins were stained with Coomassie Blue. The proteins in the remainder of the gel were transferred to nitrocellulose using the Milliblot-SDE electro-
25 blotting system (Millipore) according to manufacturer's directions. The nitrocellulose was temporarily stained with 10% Ponceau S [S.B. Carroll and A. Laughon, "Production and Purification of Polyclonal Antibodies to the Foreign Segment of β-

galactosidase Fusion Proteins," in DNA Cloning: A Practical Approach, Vol.III, (D. Glover, ed.), pp. 89-111, IRL Press, Oxford, (1987)] to visualize the lanes, then destained by running a gentle stream of distilled water over the blot for several minutes. The nitrocellulose was immersed in PBS containing 3% BSA overnight at 4°C to block any remaining protein binding sites.

The blot was cut into strips and each strip was incubated with the appropriate primary antibody. The avian anti-*C. botulinum* antibodies [described in (c)] and pre-immune chicken antibody (as control) were diluted 1:125 in PBS containing 1 mg/ml BSA for 2 hours at room temperature. The blots were washed with two changes each of large volumes of PBS, BBS-Tween and PBS, successively (10 min/wash). Goat anti-chicken IgG alkaline phosphatase conjugated secondary antibody (Fisher Biotech) was diluted 1:500 in PBS containing 1 mg/ml BSA and incubated with the blot for 2 hours at room temperature. The blots were washed with two changes each of large volumes of PBS and BBS-Tween, followed by one change of PBS and 0.1 M Tris-HCl, pH 9.5. Blots were developed in freshly prepared alkaline phosphatase substrate buffer (100 µg/ml nitroblue tetrazolium (Sigma), 50 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma), 5 mM MgCl₂ in 50 mM Na₂CO₃, pH 9.5).

The Western blots are shown in Figure 1. The anti-*C. botulinum* IgY reacted to the toxoid to give a broad immunoreactive band at about 145-150 kD on the reducing gel. This toxoid is refractive to disulfide cleavage by reducing agents due to formalin crosslinking. The immune IgY reacted with the active toxin complex, a 97 kD *C. botulinum* type A heavy chain and a 53 kD light chain. The preimmune IgY was unreactive to the *C. botulinum* complex or toxoid in the Western blot.

e) Antitoxin Antibody Titer

The IgY antibody titer to *C. botulinum* type A toxoid of eggs harvested between day 409 and 423 was evaluated by ELISA, prepared as follows. Ninety-six-well Falcon Pro-bind plates were coated overnight at 4°C with 100 µl/well toxoid [B.R. Singh & B.R. Das Gupta, Toxicon 27:403 (1989)] at 2.5 µg/ml in PBS, pH 7.5

containing 0.005% thimerosal. The following day the wells were blocked with PBS containing 1% BSA for 1 hour at 37°C. The IgY from immune or preimmune eggs was diluted in PBS containing 1% BSA and 0.05% Tween 20 and the plates were incubated for 1 hour at 37°C. The plates were washed three times with PBS
5 containing 0.05% Tween 20 and three times with PBS alone. Alkaline phosphatase-conjugated goat-anti-chicken IgG (Fisher Biotech) was diluted 1:750 in PBS containing 1% BSA and 0.05% Tween 20, added to the plates, and incubated 1 hour at 37°C. The plates were washed as before, and p-nitrophenyl phosphate (Sigma) at 1 mg/ml in 0.05 M Na₂CO₃, pH 9.5, 10 mM MgCl₂ was added.

10 The results are shown in Figure 2. Chickens immunized with the toxoid generated high titers of antibody to the immunogen. Importantly, eggs from both immunized hens had significant anti-immunogen antibody titers as compared to preimmune control eggs. The anti-*C. botulinum* IgY possessed significant activity, to a dilution of 1:93,750 or greater.

15 EXAMPLE 4

Preparation Of Avian Egg Yolk Immunoglobulin In An Orally Administrable Form

In order to administer avian IgY antibodies orally to experimental mice, an effective delivery formula for the IgY had to be determined. The concern was that if the crude IgY was dissolved in PBS, the saline in PBS would dehydrate the mice,
20 which might prove harmful over the duration of the study. Therefore, alternative methods of oral administration of IgY were tested. The example involved: (a) isolation of immune IgY; (b) solubilization of IgY in water or PBS, including subsequent dialysis of the IgY-PBS solution with water to eliminate or reduce the salts (salt and phosphate) in the buffer; and (c) comparison of the quantity and activity of recovered
25 IgY by absorbance at 280 nm and PAGE, and enzyme-linked immunoassay (ELISA).

a) Isolation Of Immune IgY

In order to investigate the most effective delivery formula for IgY, we used IgY which was raised against *Crotalus durissus terrificus* venom. Three eggs were collected from hens immunized with the *C. durissus terrificus* venom and IgY was extracted from the yolks using the modified Polson procedure described by Thalley and Carroll [Bio/Technology, 8:934-938 (1990)] as described in Example 1(c).

The egg yolks were separated from the whites, pooled, and blended with four volumes of PBS. Powdered PEG 8000 was added to a concentration of 3.5%. The mixture was centrifuged at 10,000 rpm for 10 minutes to pellet the precipitated protein, and the supernatant was filtered through cheesecloth to remove the lipid layer. Powdered PEG 8000 was added to the supernatant to bring the final PEG concentration to 12% (assuming a PEG concentration of 3.5% in the supernatant). The 12% PEG/IgY mixture was divided into two equal volumes and centrifuged to pellet the IgY.

b) Solubilization Of The IgY In Water Or PBS

One pellet was resuspended in 1/2 the original yolk volume of PBS, and the other pellet was resuspended in 1/2 the original yolk volume of water. The pellets were then centrifuged to remove any particles or insoluble material. The IgY in PBS solution dissolved readily but the fraction resuspended in water remained cloudy.

In order to satisfy anticipated sterility requirements for orally administered antibodies, the antibody solution needs to be filter-sterilized (as an alternative to heat sterilization which would destroy the antibodies). The preparation of IgY resuspended in water was too cloudy to pass through either a 0.2 or 0.45 μ m membrane filter, so 10 ml of the PBS resuspended fraction was dialyzed overnight at room temperature against 250 ml of water. The following morning the dialysis chamber was emptied and refilled with 250 ml of fresh H₂O for a second dialysis. Thereafter, the yields of soluble antibody were determined at OD₂₈₀ and are compared in Table 7.

TABLE 7

Dependence Of IgY Yield On Solvents

Fraction	Absorbance Of 1:10 Dilution At 280 nm	Percent Recovery
PBS dissolved	1.149	100%
H ₂ O dissolved	0.706	61%
PBS dissolved/H ₂ O dialyzed	0.885	77%

Resuspending the pellets in PBS followed by dialysis against water recovered more antibody than directly resuspending the pellets in water (77% versus 61%). Equivalent volumes of the IgY preparation in PBS or water were compared by PAGE, and these results were in accordance with the absorbance values (data not shown).

c) Activity Of IgY Prepared With Different Solvents

An ELISA was performed to compare the binding activity of the IgY extracted by each procedure described above. *C. durissus terrificus* (C.d.t.) venom at 2.5 µg/ml in PBS was used to coat each well of a 96-well microtiter plate. The remaining protein binding sites were blocked with PBS containing 5 mg/ml BSA. Primary antibody dilutions (in PBS containing 1 mg/ml BSA) were added in duplicate. After 2 hours of incubation at room temperature, the unbound primary antibodies were removed by washing the wells with PBS, BBS-Tween, and PBS. The species specific secondary antibody (goat anti-chicken immunoglobulin alkaline-phosphatase conjugate (Sigma) was diluted 1:750 in PBS containing 1 mg/ml BSA and added to each well of the microtiter plate. After 2 hours of incubation at room temperature, the unbound secondary antibody was removed by washing the plate as before, and freshly prepared alkaline phosphatase substrate (Sigma) at 1 mg/ml in 50 mM Na₂CO₃, 10 mM MgCl₂, pH 9.5 was added to each well. The color development was measured on a Dynatech MR 700 microplate reader using a 412 nm filter. The results are shown in Table 8.

The binding assay results parallel the recovery values in Table 7, with PBS-dissolved IgY showing slightly more activity than the PBS-dissolved/H₂O dialyzed

antibody. The water-dissolved antibody had considerably less binding activity than the other preparations.

EXAMPLE 5

Survival Of Antibody Activity After Passage Through The Gastrointestinal Tract

In order to determine the feasibility of oral administration of antibody, it was of interest to determine whether orally administered IgY survived passage through the gastrointestinal tract. The example involved: (a) oral administration of specific immune antibody mixed with a nutritional formula; and (b) assay of antibody activity extracted from feces.

TABLE 8

Antigen-Binding Activity Of IgY Prepared With Different Solvents

Dilution	Preimmune	PBS Dissolved	H ₂ O Dissolved	PBS/H ₂ O
1:500	0.005	1.748	1.577	1.742
1:2,500	0.004	0.644	0.349	0.606
1:12,500	0.001	0.144	0.054	0.090
1:62,500	0.001	0.025	0.007	0.016
1:312,500	0.010	0.000	0.000	0.002

a) Oral Administration Of Antibody

The IgY preparations used in this example are the same PBS-dissolved/H₂O dialyzed antivenom materials obtained in Example 4 above, mixed with an equal volume of Enfamil®. Two mice were used in this experiment, each receiving a different diet as follows:

- 1) water and food as usual;
- 2) immune IgY preparation dialyzed against water and mixed 1:1 with Enfamil®. (The mice were given the corresponding mixture as their only source of food and water).

b) Antibody Activity After Ingestion

After both mice had ingested their respective fluids, each tube was refilled with approximately 10 ml of the appropriate fluid first thing in the morning. By mid-morning there was about 4 to 5 ml of liquid left in each tube. At this point stool samples were collected from each mouse, weighed, and dissolved in approximately 500 μ l PBS per 100 mg stool sample. One hundred and sixty mg of control stools (no antibody) and 99 mg of experimental stools (specific antibody) in 1.5 ml microfuge tubes were dissolved in 800 and 500 μ l PBS, respectively. The samples were heated at 37°C for 10 minutes and vortexed vigorously. The experimental stools were also broken up with a narrow spatula. Each sample was centrifuged for 5 minutes in a microfuge and the supernatants, presumably containing the antibody extracts, were collected. The pellets were saved at 2-8°C in case future extracts were needed. Because the supernatants were tinted, they were diluted five-fold in PBS containing 1 mg/ml BSA for the initial dilution in the enzyme immunoassay (ELISA). The primary extracts were then diluted five-fold serially from this initial dilution. The volume of primary extract added to each well was 190 μ l. The ELISA was performed exactly as described in Example 4.

TABLE 9

Specific Antibody Activity After Passage Through The Gastrointestinal Tract

Dilution	Preimmune IgY	Control Fecal Extract	EXP. Fecal Extract
1:5	<0	0.000	0.032
1:25	0.016	<0	0.016
1:125	<0	<0	0.009
1:625	<0	0.003	0.001
1:3125	<0	<0	0.000

There was some active antibody in the fecal extract from the mouse given the specific antibody in Enfamil® formula, but it was present at a very low level. Since the samples were assayed at an initial 1:5 dilution, the binding observed could have been higher with less dilute samples. Consequently, the mice were allowed to

continue ingesting either regular food and water or the specific IgY in Enfamil® formula, as appropriate, so the assay could be repeated. Another ELISA plate was coated overnight with 5 µg/ml of *C.d.t.* venom in PBS.

5 The following morning the ELISA plate was blocked with 5 mg/ml BSA, and the fecal samples were extracted as before, except that instead of heating the extracts at 37°C, the samples were kept on ice to limit proteolysis. The samples were assayed undiluted initially, and in 5X serial dilutions thereafter. Otherwise the assay was carried out as before.

TABLE 10

10 Specific Antibody Survives Passage Through The Gastrointestinal Tract

Dilution	Preimmune IgY	Control Extract	Exp. Extract
undiluted	0.003	<0	0.379
1:5	<0	<0	0.071
1:25	0.000	<0	0.027
15 1:125	0.003	<0	0.017
1:625	0.000	<0	0.008
1:3125	0.002	<0	0.002

20 The experiment confirmed the previous results, with the antibody activity markedly higher. The control fecal extract showed no anti-*C.d.t.* activity, even undiluted, while the fecal extract from the anti-*C.d.t.* IgY/Enfamil®-fed mouse showed considerable anti-*C.d.t.* activity. This experiment (and the previous experiment) clearly demonstrate that active IgY antibody survives passage through the mouse digestive tract, a finding with favorable implications for the success of IgY antibodies administered orally as a therapeutic or prophylactic.

EXAMPLE 6

In Vivo Neutralization Of Type *C. botulinum* Type A Neurotoxin By Avian Antitoxin Antibody

This example demonstrated the ability of PEG-purified antitoxin, collected as described in Example 3, to neutralize the lethal effect of *C. botulinum* neurotoxin type A in mice. To determine the oral lethal dose (LD₁₀₀) of toxin A, groups of BALB/c mice were given different doses of toxin per unit body weight (average body weight of 24 grams). For oral administration, toxin A complex, which contains the neurotoxin associated with other non-toxin proteins was used. This complex is markedly more toxic than purified neurotoxin when given by the oral route. [I. Ohishi *et al.*, Infect. Immun., 16:106 (1977).] *C. botulinum* toxin type A complex, obtained from Eric Johnson (University Of Wisconsin, Madison) was 250 µg/ml in 50 mM sodium citrate, pH 5.5, specific toxicity 3×10^7 mouse LD₅₀/mg with parenteral administration. Approximately 40-50 ng/gm body weight was usually fatal within 48 hours in mice maintained on conventional food and water. When mice were given a diet *ad libitum* of only Enfamil® the concentration needed to produce lethality was approximately 2.5 times higher (125 ng/gm body weight). Botulinal toxin concentrations of approximately 200 ng/gm body weight were fatal in mice fed Enfamil® containing preimmune IgY (resuspended in Enfamil® at the original yolk volume).

The oral LD₁₀₀ of *C. botulinum* toxin was also determined in mice that received known amounts of a mixture of preimmune IgY-Ensure® delivered orally through feeding needles. Using a 22 gauge feeding needle, mice were given 250 µl each of a preimmune IgY-Ensure® mixture (preimmune IgY dissolved in 1/4 original yolk volume) 1 hour before and 1/2 hour and 5 hours after administering botulinal toxin. Toxin concentrations given orally ranged from approximately 12 to 312 ng/gm body weight (0.3 to 7.5 µg per mouse). Botulinal toxin complex concentration of approximately 40 ng/gm body weight (1 µg per mouse) was lethal in all mice in less than 36 hours.

Two groups of BALB/c mice, 10 per group, were each given orally a single dose of 1 µg each of botulinal toxin complex in 100 µl of 50 mM sodium citrate pH 5.5. The mice received 250 µl treatments of a mixture of either preimmune or immune IgY in Ensure® (1/4 original yolk volume) 1 hour before and 1/2 hour, 4 hours, and 8 hours after botulinal toxin administration. The mice received three treatments per day for two more days. The mice were observed for 96 hours. The survival and mortality are shown in Table 11.

TABLE 11

Neutralization Of Botulinal Toxin A *In Vivo*

Toxin Dose ng/gm	Antibody Type	Number Of Mice Alive	Number Of Mice Dead
41.6	non-immune	0	10
41.6	anti-botulinal toxin	10	0

All mice treated with the preimmune IgY-Ensure® mixture died within 46 hours post-toxin administration. The average time of death in the mice was 32 hours post toxin administration. Treatments of preimmune IgY-Ensure® mixture did not continue beyond 24 hours due to extensive paralysis of the mouth in mice of this group. In contrast, all ten mice treated with the immune anti-botulinal toxin IgY-Ensure® mixture survived past 96 hours. Only 4 mice in this group exhibited symptoms of botulism toxicity (two mice about 2 days after and two mice 4 days after toxin administration). These mice eventually died 5 and 6 days later. Six of the mice in this immune group displayed no adverse effects to the toxin and remained alive and healthy long term. Thus, the avian anti-botulinal toxin antibody demonstrated very good protection from the lethal effects of the toxin in the experimental mice.

EXAMPLE 7

Production Of An Avian Antitoxin Against *Clostridium difficile* Toxin A

Toxin A is a potent cytotoxin secreted by pathogenic strains of *C. difficile*, that plays a direct role in damaging gastrointestinal tissues. In more severe cases of *C. difficile* intoxication, pseudomembranous colitis can develop which may be fatal. This would be prevented by neutralizing the effects of this toxin in the gastrointestinal tract. As a first step, antibodies were produced against a portion of the toxin. The example involved: (a) conjugation of a synthetic peptide of toxin A to bovine serum albumin; (b) immunization of hens with the peptide-BSA conjugate; and (c) detection of antitoxin peptide antibodies by ELISA.

a) Conjugation Of A Synthetic Peptide Of Toxin A To Bovine Serum Albumin

The synthetic peptide CQTIDGKKYYFN-NH₂ (SEQ ID NO:82) was prepared commercially (Multiple Peptide Systems, San Diego, CA) and validated to be >80% pure by high-pressure liquid chromatography. The eleven amino acids following the cysteine residue represent a consensus sequence of a repeated amino acid sequence found in Toxin A. [Wren *et al.*, Infect. Immun., 59:3151-3155 (1991).] The cysteine was added to facilitate conjugation to carrier protein.

In order to prepare the carrier for conjugation, BSA (Sigma) was dissolved in 0.01 M NaPO₄, pH 7.0 to a final concentration of 20 mg/ml and n-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Pierce) was dissolved in N,N-dimethyl formamide to a concentration of 5 mg/ml. MBS solution, 0.51 ml, was added to 3.25 ml of the BSA solution and incubated for 30 minutes at room temperature with stirring every 5 minutes. The MBS-activated BSA was then purified by chromatography on a Bio-Gel P-10 column (Bio-Rad; 40 ml bed volume) equilibrated with 50 mM NaPO₄, pH 7.0 buffer. Peak fractions were pooled (6.0 ml).

Lyophilized toxin A peptide (20 mg) was added to the activated BSA mixture, stirred until the peptide dissolved and incubated 3 hours at room temperature. Within 20 minutes, the reaction mixture became cloudy and precipitates formed. After 3 hours, the reaction mixture was centrifuged at $10,000 \times g$ for 10 min and the supernatant analyzed for protein content. No significant protein could be detected at 280 nm. The conjugate precipitate was washed three times with PBS and stored at 4°C. A second conjugation was performed with 15 mg of activated BSA and 5 mg of peptide and the conjugates pooled and suspended at a peptide concentration of 10 mg/ml in 10 mM NaPO_4 , pH 7.2.

b) Immunization Of Hens With Peptide Conjugate

Two hens were each initially immunized on day zero by injection into two subcutaneous and two intramuscular sites with 1 mg of peptide conjugate that was emulsified in CFA (GIBCO). The hens were boosted on day 14 and day 21 with 1 mg of peptide conjugate emulsified in IFA (GIBCO).

c) Detection Of Antitoxin Peptide Antibodies By ELISA

IgY was purified from two eggs obtained before immunization (pre-immune) and two eggs obtained 31 and 32 days after the initial immunization using PEG fractionation as described in Example 1.

Wells of a 96-well microtiter plate (Falcon Pro-Bind Assay Plate) were coated overnight at 4°C with 100 $\mu\text{g}/\text{ml}$ solution of the toxin A synthetic peptide in PBS, pH 7.2 prepared by dissolving 1 mg of the peptide in 1.0 ml of H_2O and dilution of PBS. The pre-immune and immune IgY preparations were diluted in a five-fold series in a buffer containing 1% PEG 8000 and 0.1% Tween-20 (v/v) in PBS, pH 7.2. The wells were blocked for 2 hours at room temperature with 150 μl of a solution containing 5% (v/v) Carnation® nonfat dry milk and 1% PEG 8000 in PBS, pH 7.2. After incubation for 2 hours at room temperature, the wells were washed, secondary rabbit anti-chicken IgG-alkaline phosphatase (1:750) added, the wells washed again and the

color development obtained as described in Example 1. The results are shown in Table 12.

TABLE 12
Reactivity Of IgY With Toxin Peptide

Dilution Of PEG Prep	Absorbance At 410 nm	
	Preimmune	Immune Anti-Peptide
1:100	0.013	0.253
1:500	0.004	0.039
1:2500	0.004	0.005

Clearly, the immune antibodies contain titers against this repeated epitope of toxin A.

EXAMPLE 8

Production Of Avian Antitoxins Against *Clostridium difficile* Native Toxins A And B

To determine whether avian antibodies are effective for the neutralization of *C. difficile* toxins, hens were immunized using native *C. difficile* toxins A and B. The resulting egg yolk antibodies were then extracted and assessed for their ability to neutralize toxins A and B *in vitro*. The Example involved (a) preparation of the toxin immunogens, (b) immunization, (c) purification of the antitoxins, and (d) assay of toxin neutralization activity.

a) Preparation Of The Toxin Immunogens

Both *C. difficile* native toxins A and B, and *C. difficile* toxoids, prepared by the treatment of the native toxins with formaldehyde, were employed as immunogens. *C. difficile* toxoids A and B were prepared by a procedure which was modified from published methods (Ehrich *et al.*, Infect. Immun. 28:1041 (1980). Separate solutions (in PBS) of native *C. difficile* toxin A and toxin B (Tech Lab) were each adjusted to a

concentration of 0.20 mg/ml, and formaldehyde was added to a final concentration of 0.4%. The toxin/formaldehyde solutions were then incubated at 37°C for 40 hrs. Free formaldehyde was then removed from the resulting toxoid solutions by dialysis against PBS at 4°C. In previously published reports, this dialysis step was not performed.

5 Therefore, free formaldehyde must have been present in their toxoid preparations. The toxoid solutions were concentrated, using a Centriprep concentrator unit (Amicon), to a final toxoid concentration of 4.0 mg/ml. The two resulting preparations were designated as toxoid A and toxoid B.

10 *C. difficile* native toxins were prepared by concentrating stock solutions of toxin A and toxin B (Tech Lab, Inc), using Centriprep concentrator units (Amicon), to a final concentration of 4.0 mg/ml.

b) Immunization

The first two immunizations were performed using the toxoid A and toxoid B immunogens described above. A total of 3 different immunization combinations were employed. For the first immunization group, 0.2 ml of toxoid A was emulsified in an equal volume of Titer Max adjuvant (CytRx). Titer Max was used in order to conserve the amount of immunogen used, and to simplify the immunization procedure. This immunization group was designated "CTA." For the second immunization group, 0.1 ml of toxoid B was emulsified in an equal volume of Titer Max adjuvant. This group was designated "CTB." For the third immunization group, 0.2 ml of toxoid A was first mixed with 0.2 ml of toxoid B, and the resulting mixture was emulsified in 0.4 ml of Titer Max adjuvant. This group was designated "CTAB." In this way, three separate immunogen emulsions were prepared, with each emulsion containing a final concentration of 2.0 mg/ml of toxoid A (CTA) or toxoid B (CTB) or a mixture of 2.0 mg/ml toxoid A and 2.0 mg/ml toxoid B (CTAB).

On day 0, White Leghorn hens, obtained from a local breeder, were immunized as follows: Group CTA. Four hens were immunized, with each hen receiving 200µg of toxoid A, via two intramuscular (I.M.) injections of 50µl of CTA emulsion in the breast area. Group CTB. One hen was immunized with 200µg of toxoid B, via two

I.M. injections of 50µl of CTB emulsion in the breast area. **Gr up CTAB.** Four hens were immunized, with each hen receiving a mixture containing 200µg of toxoid A and 200µg of toxoid B, via two I.M. injections of 100µl of CTAB emulsion in the breast area. The second immunization was performed 5 weeks later, on day 35,
5 exactly as described for the first immunization above.

In order to determine whether hens previously immunized with *C. difficile* toxoids could tolerate subsequent booster immunizations using native toxins, a single hen from group CTAB was immunized for a third time, this time using a mixture of the native toxin A and native toxin B described in section (a) above (these toxins were
10 not formaldehyde-treated, and were used in their active form). This was done in order to increase the amount (titer) and affinity of specific antitoxin antibody produced by the hen over that achieved by immunizing with toxoids only. On day 62, 0.1 ml of a toxin mixture was prepared which contained 200µg of native toxin A and 200µg of native toxin B. This toxin mixture was then emulsified in 0.1 ml of Titer Max
15 adjuvant. A single CTAB hen was then immunized with the resulting immunogen emulsion, via two I.M. injections of 100µl each, into the breast area. This hen was marked with a wing band, and observed for adverse effects for a period of approximately 1 week, after which time the hen appeared to be in good health.

Because the CTAB hen described above tolerated the booster immunization
20 with native toxins A and B with no adverse effects, it was decided to boost the remaining hens with native toxin as well. On day 70, booster immunizations were performed as follows: **Group CTA.** A 0.2 ml volume of the 4 mg/ml native toxin A solution was emulsified in an equal volume of Titer Max adjuvant. Each of the 4 hens was then immunized with 200µg of native toxin A, as described for the toxoid A
25 immunizations above. **Group CTB.** A 50µl volume of the 4 mg/ml native toxin B solution was emulsified in an equal volume of Titer Max adjuvant. The hen was then immunized with 200µg of native toxin B, as described for the toxoid B immunizations above. **Group CTAB.** A 0.15 ml volume of the 4 mg/ml native toxin A solution was first mixed with a 0.15 ml volume the 4 mg/ml native toxin B solution. The resulting
30 toxin mixture was then emulsified in 0.3 ml of Titer Max adjuvant. The 3 remaining

hens (the hen with the wing band was not immunized this time) were then immunized with 200µg of native toxin A and 200µg of native toxin B as described for the toxoid A+ toxoid B immunizations (CTAB) above. On day 85, all hens received a second booster immunization using native toxins, done exactly as described for the first boost with native toxins above.

All hens tolerated both booster immunizations with native toxins with no adverse effects. As previous literature references describe the use of formaldehyde-treated toxoids, this is apparently the first time that any immunizations have been performed using native *C. difficile* toxins.

c) Purification Of Antitoxins

Eggs were collected from the hen in group CTB 10-12 days following the second immunization with toxoid (day 35 immunization described in section (b) above), and from the hens in groups CTA and CTAB 20-21 days following the second immunization with toxoid. To be used as a pre-immune (negative) control, eggs were also collected from unimmunized hens from the same flock. Egg yolk immunoglobulin (IgY) was extracted from the 4 groups of eggs as described in Example 1 (c), and the final IgY pellets were solubilized in the original yolk volume of PBS without thimerosal. Importantly, thimerosal was excluded because it would have been toxic to the CHO cells used in the toxin neutralization assays described in section (d) below.

d) Assay Of Toxin Neutralization Activity

The toxin neutralization activity of the IgY solutions prepared in section (c) above was determined using an assay system that was modified from published methods. [Ehrich *et al.*, Infect. Immun. 28:1041-1043 (1992); and McGee *et al.* Microb. Path. 12:333-341 (1992).] As additional controls, affinity-purified goat anti-*C. difficile* toxin A (Tech Lab) and affinity-purified goat anti-*C. difficile* toxin B (Tech Lab) were also assayed for toxin neutralization activity.

The IgY solutions and goat antibodies were serially diluted using F 12 medium (GIBCO) which was supplemented with 2% FCS (GIBCO)(this solution will be referred to as "medium" for the remainder of this Example). The resulting antibody solutions were then mixed with a standardized concentration of either native *C. difficile* toxin A (Tech Lab), or native *C. difficile* toxin B (Tech Lab), at the concentrations indicated below. Following incubation at 37°C for 60 min., 100µl volumes of the toxin + antibody mixtures were added to the wells of 96-well microtiter plates (Falcon Microtest III) which contained 2.5×10^4 Chinese Hamster Ovary (CHO) cells per well (the CHO cells were plated on the previous day to allow them to adhere to the plate wells). The final concentration of toxin, or dilution of antibody indicated below refers to the final test concentration of each reagent present in the respective microtiter plate wells. Toxin reference wells were prepared which contained CHO cells and toxin A or toxin B at the same concentration used for the toxin plus antibody mixtures (these wells contained no antibody). Separate control wells were also prepared which contained CHO cells and medium only. The assay plates were then incubated for 18-24 hrs. in a 37°C, humidified, 5% CO₂ incubator. On the following day, the remaining adherent (viable) cells in the plate wells were stained using 0.2% crystal violet (Mallinckrodt) dissolved in 2% ethanol, for 10 min. Excess stain was then removed by rinsing with water, and the stained cells were solubilized by adding 100µl of 1% SDS (dissolved in water) to each well. The absorbance of each well was then measured at 570 nm, and the percent cytotoxicity of each test sample or mixture was calculated using the following formula:

$$\% \text{ CHO Cell Cytotoxicity} = [1 - (\frac{\text{Abs. Sample}}{\text{Abs. Control}})] \times 100$$

Unlike previous reports which quantitate results visually by counting cell rounding by microscopy, this Example utilized spectrophotometric methods to quantitate the *C. difficile* toxin bioassay. In order to determine the toxin A

neutralizing activity of the CTA, CTAB, and pre-immune IgY preparations, as well as the affinity-purified goat antitoxin A control, dilutions of these antibodies were reacted against a 0.1µg/ml concentration of native toxin A (this is the approx. 50% cytotoxic dose of toxin A in this assay system). The results are shown in Figure 3.

5 Complete neutralization of toxin A occurred with the CTA IgY (antitoxin A, above) at dilutions of 1:80 and lower, while significant neutralization occurred out to the 1:320 dilution. The CTAB IgY (antitoxin A + toxin B, above) demonstrated complete neutralization at the 1:320-1:160 and lower dilutions, and significant neutralization occurred out to the 1:1280 dilution. The commercially available
10 affinity-purified goat antitoxin A did not completely neutralize toxin A at any of the dilutions tested, but demonstrated significant neutralization out to a dilution of 1:1,280. The preimmune IgY did not show any toxin A neutralizing activity at any of the concentrations tested. These results demonstrate that IgY purified from eggs laid by hens immunized with toxin A alone, or simultaneously with toxin A and toxin B, is an
15 effective toxin A antitoxin.

The toxin B neutralizing activity of the CTAB and pre-immune IgY preparations, and also the affinity-purified goat antitoxin B control was determined by reacting dilutions of these antibodies against a concentration of native toxin B of 0.1 ng/ml (approximately the 50% cytotoxic dose of toxin B in the assay system). The
20 results are shown in Figure 4.

Complete neutralization of toxin B occurred with the CTAB IgY (antitoxin A + toxin B, above) at the 1:40 and lower dilutions, and significant neutralization occurred out to the 1:320 dilution. The affinity-purified goat antitoxin B demonstrated complete neutralization at dilutions of 1:640 and lower, and significant neutralization occurred
25 out to a dilution of 1:2,560. The preimmune IgY did not show any toxin B neutralizing activity at any of the concentrations tested. These results demonstrate that IgY purified from eggs laid by hens immunized simultaneously with toxin A and toxin B is an effective toxin B antitoxin.

In a separate study, the toxin B neutralizing activity of CTB, CTAB, and pre-immune IgY preparations was determined by reacting dilutions of these antibodies
30

against a native toxin B concentration of 0.1 µg/ml (approximately 100% cytotoxic dose of toxin B in this assay system). The results are shown in Figure 5.

Significant neutralization of toxin B occurred with the CTB IgY (antitoxin B, above) at dilutions of 1:80 and lower, while the CTAB IgY (antitoxin A + toxin B, above) was found to have significant neutralizing activity at dilutions of 1:40 and lower. The preimmune IgY did not show any toxin B neutralizing activity at any of the concentrations tested. These results demonstrate that IgY purified from eggs laid by hens immunized with toxin B alone, or simultaneously with toxin A and toxin B, is an effective toxin B antitoxin.

EXAMPLE 9

In vivo Protection Of Golden Syrian Hamsters From *C. difficile* Disease By Avian Antitoxins Against *C. difficile* Toxins A And B

The most extensively used animal model to study *C. difficile* disease is the hamster. [Lyerly *et al.*, Infect. Immun. 47:349-352 (1992).] Several other animal models for antibiotic-induced diarrhea exist, but none mimic the human form of the disease as closely as the hamster model. [R. Fekety, "Animal Models of Antibiotic-Induced Colitis," in O. Zak and M. Sande (eds.), *Experimental Models in Antimicrobial Chemotherapy*, Vol. 2, pp.61-72, (1986).] In this model, the animals are first predisposed to the disease by the oral administration of an antibiotic, such as clindamycin, which alters the population of normally-occurring gastrointestinal flora (Fekety, at 61-72). Following the oral challenge of these animals with viable *C. difficile* organisms, the hamsters develop cecitis, and hemorrhage, ulceration, and inflammation are evident in the intestinal mucosa. [Lyerly *et al.*, Infect. Immun. 47:349-352 (1985).] The animals become lethargic, develop severe diarrhea, and a high percentage of them die from the disease. [Lyerly *et al.*, Infect. Immun. 47:349-352 (1985).] This model is therefore ideally suited for the evaluation of therapeutic agents designed for the treatment or prophylaxis of *C. difficile* disease.

The ability of the avian *C. difficile* antitoxins, described in Example 1 above, to protect hamsters from *C. difficile* disease was evaluated using the Golden Syrian hamster model of *C. difficile* infection. The Example involved (a) preparation of the avian *C. difficile* antitoxins, (b) *in vivo* protection of hamsters from *C. difficile* disease by treatment with avian antitoxins, and (c) long-term survival of treated hamsters.

a) Preparation Of The Avian *C. difficile* Antitoxins

Eggs were collected from hens in groups CTA and CTAB described in Example 1 (b) above. To be used as a pre-immune (negative) control, eggs were also purchased from a local supermarket. Egg yolk immunoglobulin (IgY) was extracted from the 3 groups of eggs as described in Example 1 (c), and the final IgY pellets were solubilized in one fourth the original yolk volume of Ensure® nutritional formula.

**b) *In vivo* Protection Of Hamsters Against *C. difficile*
Disease By Treatment With Avian Antitoxins**

The avian *C. difficile* antitoxins prepared in section (a) above were evaluated for their ability to protect hamsters from *C. difficile* disease using an animal model system which was modified from published procedures. [Fekety, at 61-72; Borriello *et al.*, J. Med. Microbiol., 24:53-64 (1987); Kim *et al.*, Infect. Immun., 55:2984-2992 (1987); Borriello *et al.*, J. Med. Microbiol., 25:191-196 (1988); Delmee and Avesani, J. Med. Microbiol., 33:85-90 (1990); and Lysterly *et al.*, Infect. Immun., 59:2215-2218 (1991).] For the study, three separate experimental groups were used, with each group consisting of 7 female Golden Syrian hamsters (Charles River), approximately 10 weeks old and weighing approximately 100 gms. each. The three groups were designated "CTA," "CTAB" and "Pre-immune." These designations corresponded to the antitoxin preparations with which the animals in each group were treated. Each animal was housed in an individual cage, and was offered food and water *ad libitum* through the entire length of the study. On day 1, each animal was orally administered

1.0 ml of one of the three antitoxin preparations (prepared in section (a) above) at the following timepoints: 0 hrs., 4 hrs., and 8 hrs. On day 2, the day 1 treatment was repeated. On day 3, at the 0 hr. timepoint, each animal was again administered antitoxin, as described above. At 1 hr., each animal was orally administered 3.0 mg of clindamycin-HCl (Sigma) in 1 ml of water. This treatment predisposed the animals to infection with *C. difficile*. As a control for possible endogenous *C. difficile* colonization, an additional animal from the same shipment (untreated) was also administered 3.0 mg of clindamycin-HCl in the same manner. This clindamycin control animal was left untreated (and uninfected) for the remainder of the study. At the 4 hr. and 8 hr. timepoints, the animals were administered antitoxin as described above. On day 4, at the 0 hr. timepoint, each animal was again administered antitoxin as described above. At 1 hr., each animal was orally challenged with 1 ml of *C. difficile* inoculum, which contained approx. 100 *C. difficile* strain 43596 organisms in sterile saline. *C. difficile* strain 43596, which is a serogroup C strain, was chosen because it is representative of one of the most frequently-occurring serogroups isolated from patients with antibiotic-associated pseudomembranous colitis. [Delmee *et al.*, J. Clin. Microbiol., 28:2210-2214 (1985).] In addition, this strain has been previously demonstrated to be virulent in the hamster model of infection. [Delmee and Avesani, J. Med. Microbiol., 33:85-90 (1990).] At the 4 hr. and 8 hr. timepoints, the animals were administered antitoxin as described above. On days 5 through 13, the animals were administered antitoxin 3x per day as described for day 1 above, and observed for the onset of diarrhea and death. On the morning of day 14, the final results of the study were tabulated. These results are shown in Table 13.

Representative animals from those that died in the Pre-Immune and CTA groups were necropsied. Viable *C. difficile* organisms were cultured from the ceca of these animals, and the gross pathology of the gastrointestinal tracts of these animals was consistent with that expected for *C. difficile* disease (inflamed, distended, hemorrhagic cecum, filled with watery diarrhea-like material). In addition, the clindamycin control animal remained healthy throughout the entire study period,

therefore indicating that the hamsters used in the study had not previously been colonized with endogenous *C. difficile* organisms prior to the start of the study. Following the final antitoxin treatment on day 13, a single surviving animal from the CTA group, and also from the CTAB group, was sacrificed and necropsied. No pathology was noted in either animal.

TABLE 13

Treatment Results

Treatment Group	No. Animals Surviving	No. Animals Dead
Pre-Immune	1	6
CTA (Antitoxin A only)	5	2
CTAB (Antitoxin A + Antitoxin B)	7	0

Treatment of hamsters with orally-administered toxin A and toxin B antitoxin (group CTAB) successfully protected 7 out of 7 (100%) of the animals from *C. difficile* disease. Treatment of hamsters with orally-administered toxin A antitoxin (group CTA) protected 5 out of 7 (71%) of these animals from *C. difficile* disease. Treatment using pre-immune IgY was not protective against *C. difficile* disease, as only 1 out of 7 (14%) of these animals survived. These results demonstrate that the avian toxin A antitoxin and the avian toxin A + toxin B antitoxin effectively protected the hamsters from *C. difficile* disease. These results also suggest that although the neutralization of toxin A alone confers some degree of protection against *C. difficile* disease, in order to achieve maximal protection, simultaneous antitoxin A and antitoxin B activity is necessary.

c) Long-Term Survival Of Treated Hamsters

It has been previously reported in the literature that hamsters treated with orally-administered bovine antitoxin IgG concentrate are protected from *C. difficile* disease as long as the treatment is continued, but when the treatment is stopped, the animals develop diarrhea and subsequently die within 72 hrs. [Lyerly *et al.*, Infect. Immun., 59(6):2215-2218 (1991).]

In order to determine whether treatment of *C. difficile* disease using avian antitoxins promotes long-term survival following the discontinuation of treatment, the 4 surviving animals in group CTA, and the 6 surviving animals in group CTAB were observed for a period of 11 days (264 hrs.) following the discontinuation of antitoxin treatment described in section (b) above. All hamsters remained healthy through the entire post-treatment period. This result demonstrates that not only does treatment with avian antitoxin protect against the onset of *C. difficile* disease (*i.e.*, it is effective as a prophylactic), it also promotes long-term survival beyond the treatment period, and thus provides a lasting cure.

EXAMPLE 10

In vivo Treatment Of Established *C. difficile* Infection In Golden Syrian Hamsters With Avian Antitoxins Against *C. difficile* Toxins A And B

The ability of the avian *C. difficile* antitoxins, described in Example 8 above, to treat an established *C. difficile* infection was evaluated using the Golden Syrian hamster model. The Example involved (a) preparation of the avian *C. difficile* antitoxins, (b) *in vivo* treatment of hamsters with established *C. difficile* infection, and (c) histologic evaluation of cecal tissue.

a) Preparation Of The Avian *C. difficile* Antitoxins

Eggs were collected from hens in group CTAB described in Example 8 (b) above, which were immunized with *C. difficile* toxoids and native toxins A and B. Eggs purchased from a local supermarket were used as a pre-immune (negative) control. Egg yolk immunoglobulin (IgY) was extracted from the 2 groups of eggs as described in Example 1 (c), and the final IgY pellets were solubilized in one-fourth the original yolk volume of Ensure® nutritional formula.

**b) In vivo Treatment Of Hamsters With Established
C. difficile Infection**

The avian *C. difficile* antitoxins prepared in section (a) above were evaluated for the ability to treat established *C. difficile* infection in hamsters using an animal model system which was modified from the procedure which was described for the hamster protection study in Example 8(b) above.

For the study, four separate experimental groups were used, with each group consisting of 7 female Golden Syrian hamsters (Charles River), approx. 10 weeks old, weighing approximately 100 gms. each. Each animal was housed separately, and was offered food and water *ad libitum* through the entire length of the study.

On day 1 of the study, the animals in all four groups were each predisposed to *C. difficile* infection by the oral administration of 3.0 mg of clindamycin-HCl (Sigma) in 1 ml of water.

On day 2, each animal in all four groups was orally challenged with 1 ml of *C. difficile* inoculum, which contained approximately 100 *C. difficile* strain 43596 organisms in sterile saline. *C. difficile* strain 43596 was chosen because it is representative of one of the most frequently-occurring serogroups isolated from patients with antibiotic-associated pseudomembranous colitis. [Delmee *et al.*, J. Clin. Microbiol., 28:2210-2214 (1990).] In addition, as this was the same *C. difficile* strain used in all of the previous Examples above, it was again used in order to provide experimental continuity.

On day 3 of the study (24 hrs. post-infection), treatment was started for two of the four groups of animals. Each animal of one group was orally administered 1.0 ml of the CTAB IgY preparation (prepared in section (a) above) at the following timepoints: 0 hrs., 4 hrs., and 8 hrs. The animals in this group were designated "CTAB-24." The animals in the second group were each orally administered 1.0 ml of the pre-immune IgY preparation (also prepared in section (a) above) at the same timepoints as for the CTAB group. These animals were designated "Pre-24." Nothing was done to the remaining two groups of animals on day 3.

On day 4, 48 hrs. post-infection, the treatment described for day 3 above was repeated for the CTAB-24 and Pre-24 groups, and was initiated for the remaining two groups at the same timepoints. The final two groups of animals were designated "CTAB-48" and "Pre-48" respectively.

On days 5 through 9, the animals in all four groups were administered antitoxin or pre-immune IgY, 3x per day, as described for day 4 above. The four experimental groups are summarized in Table 14.

TABLE 14

Experimental Treatment Groups

Group Designation	Experimental Treatment
CTAB-24	Infected, treatment w/antitoxin IgY started @ 24 hrs. post-infection.
Pre-24	Infected, treatment w/pre-immune IgY started @ 24 hrs. post-infection.
CTAB-48	Infected, treatment w/antitoxin IgY started @ 48 hrs. post-infection.
Pre-48	Infected, treatment w/pre-immune IgY started @ 48 hrs. post-infection.

All animals were observed for the onset of diarrhea and death through the conclusion of the study on the morning of day 10. The results of this study are displayed in Table 15.

TABLE 15

Experimental Outcome--Day 10

Treatment Group	No. Animals Surviving	No. Animals Dead
CTAB-24	6	1
Pre-24	0	7
CTAB-48	4	3
Pre-48	2	5

Eighty-six percent of the animals which began receiving treatment with antitoxin IgY at 24 hrs. post-infection (CTAB-24 above) survived, while 57% of the animals treated with antitoxin IgY starting 48 hrs. post-infection (CTAB-48 above) survived. In contrast, none of the animals receiving pre-immune IgY starting 24 hrs.

post-infection (Pre-24 above) survived, and only 29% of the animals which began receiving treatment with pre-immune IgY at 48 hrs. post-infection (Pre-48 above) survived through the conclusion of the study. These results demonstrate that avian antitoxins raised against *C. difficile* toxins A and B are capable of successfully treating established *C. difficile* infections *in vivo*.

c) Histologic Evaluation Of Cecal Tissue

In order to further evaluate the ability of the IgY preparations tested in this study to treat established *C. difficile* infection, histologic evaluations were performed on cecal tissue specimens obtained from representative animals from the study described in section (b) above.

Immediately following death, cecal tissue specimens were removed from animals which died in the Pre-24 and Pre-48 groups. Following the completion of the study, a representative surviving animal was sacrificed and cecal tissue specimens were removed from the CTAB-24 and CTAB-48 groups. A single untreated animal from the same shipment as those used in the study was also sacrificed and a cecal tissue specimen was removed as a normal control. All tissue specimens were fixed overnight at 4°C in 10% buffered formalin. The fixed tissues were paraffin-embedded, sectioned, and mounted on glass microscope slides. The tissue sections were then stained using hematoxylin and eosin (H and E stain), and were examined by light microscopy.

Upon examination, the tissues obtained from the CTAB-24 and CTAB-48 animals showed no pathology, and were indistinguishable from the normal control. This observation provides further evidence for the ability of avian antitoxins raised against *C. difficile* toxins A and B to effectively treat established *C. difficile* infection, and to prevent the pathologic consequences which normally occur as a result of *C. difficile* disease.

In contrast, characteristic substantial mucosal damage and destruction was observed in the tissues of the animals from the Pre-24 and Pre-48 groups which died

from *C. difficile* disease. Normal tissue architecture was obliterated in these two preparations, as most of the mucosal layer was observed to have sloughed away, and there were numerous large hemorrhagic areas containing massive numbers of erythrocytes.

5

EXAMPLE 11

Cloning And Expression Of *C. difficile* Toxin A Fragments

The toxin A gene has been cloned and sequenced, and shown to encode a protein of predicted MW of 308 kd. [Dove *et al.*, Infect. Immun., 58:480-488 (1990).] Given the expense and difficulty of isolating native toxin A protein, it would be
10 advantageous to use simple and inexpensive procaryotic expression systems to produce and purify high levels of recombinant toxin A protein for immunization purposes. Ideally, the isolated recombinant protein would be soluble in order to preserve native antigenicity, since solubilized inclusion body proteins often do not fold into native conformations. To allow ease of purification, the recombinant protein should be
15 expressed to levels greater than 1 mg/liter of *E. coli* culture.

To determine whether high levels of recombinant toxin A protein can be produced in *E. coli*, fragments of the toxin A gene were cloned into various prokaryotic expression vectors, and assessed for the ability to express recombinant toxin A protein in *E. coli*. Three prokaryotic expression systems were utilized. These
20 systems were chosen because they drive expression of either fusion (pMALc and pGEX2T) or native (pET23a-c) protein to high levels in *E. coli*, and allow affinity purification of the expressed protein on a ligand containing column. Fusion proteins expressed from pGEX vectors bind glutathione agarose beads, and are eluted with reduced glutathione. pMAL fusion proteins bind amylose resin, and are eluted with
25 maltose. A poly-histidine tag is present at either the N-terminal (pET16b) or C-terminal (pET23a-c) end of pET fusion proteins. This sequence specifically binds Ni_2^+ chelate columns, and is eluted with imidazole salts. Extensive descriptions of these

vectors are available [Williams *et al.* (1995) *DNA Cloning 2: Expression Systems*, Glover and Hames, eds. IRL Press, Oxford, pp. 15-58], and will not be discussed in detail here. The Example involved (a) cloning of the toxin A gene, (b) expression of large fragments of toxin A in various prokaryotic expression systems, (c) identification of smaller toxin A gene fragments that express efficiently in *E. coli*, (d) purification of recombinant toxin A protein by affinity chromatography, and (e) demonstration of functional activity of a recombinant fragment of the toxin A gene.

a) Cloning Of The Toxin A Gene

A restriction map of the toxin A gene is shown in Figure 6. The encoded protein contains a carboxy terminal ligand binding region, containing multiple repeats of a carbohydrate binding domain. [von Eichel-Streiber and Sauerborn, *Gene* 96:107-113 (1990).] The toxin A gene was cloned in three pieces, by using either the polymerase chain reaction (PCR) to amplify specific regions, (regions 1 and 2, Figure 6) or by screening a constructed genomic library for a specific toxin A gene fragment (region 3, Figure 6). The sequences of the utilized PCR primers are P1: 5' GGAAATTAGCTGCAGCATCTGAC 3' (SEQ ID NO.:1); P2: 5' TCTAGCAAATTCGCTTGTGTTGAA 3' (SEQ ID NO.:2); P3: 5' CTCGCATATAGCATTAGACC 3' (SEQ ID NO.:3); and P4: 5' CTATCTAGGCCTAAAGTAT 3' (SEQ ID NO.:4). These regions were cloned into prokaryotic expression vectors that express either fusion (pMALc and pGEX2T) or native (pET23a-c) protein to high levels in *E. coli*, and allow affinity purification of the expressed protein on a ligand containing column.

Clostridium difficile VPI strain 10463 was obtained from the ATCC (ATCC #43255) and grown under anaerobic conditions in brain-heart infusion medium (BBL). High molecular-weight *C. difficile* DNA was isolated essentially as described by Wren and Tabaqchali (1987) *J. Clin. Microbiol.*, 25:2402, except proteinase K and sodium dodecyl sulfate (SDS) was used to disrupt the bacteria, and cetyltrimethylammonium bromide precipitation [as described in Ausubel *et al.*, *Current Protocols in Molecular Biology* (1989)] was used to remove carbohydrates from the cleared lysate. The

integrity and yield of genomic DNA was assessed by comparison with a serial dilution of uncut lambda DNA after electrophoresis on an agarose gel.

Fragments 1 and 2 were cloned by PCR, utilizing a proofreading thermostable DNA polymerase (native *pfu* polymerase; Stratagene). The high fidelity of this polymerase reduces the mutation problems associated with amplification by error prone polymerases (*e.g.*, *Taq* polymerase). PCR amplification was performed using the indicated PCR primers (Figure 6) in 50 µl reactions containing 10 mM Tris-HCl(8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 µM each dNTP, 0.2 µM each primer, and 50 ng *C. difficile* genomic DNA. Reactions were overlaid with 100 µl mineral oil, heated to 94°C for 4 min, 0.5 µl native *pfu* polymerase (Stratagene) added, and the reaction cycled 30x at 94°C for 1 min, 50°C for 1 min, 72°C for 4 min, followed by 10 min at 72°C. Duplicate reactions were pooled, chloroform extracted, and ethanol precipitated. After washing in 70% ethanol, the pellets were resuspended in 50 µl TE buffer [10 mM Tris-HCL, 1 mM EDTA pH 8.0]. Aliquots of 10µl each were restriction digested with either *EcoRI/HincII* (fragment 1) or *EcoRI/PstI* (fragment 2), and the appropriate restriction fragments were gel purified using the Prep-A-Gene kit (BioRad), and ligated to either *EcoRI/SmaI*-restricted pGEX2T (Pharmacia) vector (fragment 1), or the *EcoRI/PstI* pMALc (New England Biolabs) vector (fragment 2). Both clones are predicted to produce in-frame fusions with either the glutathione-S-transferase protein (pGEX vector) or the maltose binding protein (pMAL vector). Recombinant clones were isolated, and confirmed by restriction digestion, using standard recombinant molecular biology techniques. [Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (1989), and designated pGA30-660 and pMA660-1100, respectively (*see* Figure 6 for description of the clone designations).]

Fragment 3 was cloned from a genomic library of size selected *PstI* digested *C. difficile* genomic DNA, using standard molecular biology techniques (Sambrook *et al.*). Given that the fragment 3 internal *PstI* site is protected from cleavage in *C. difficile* genomic DNA [Price *et al.*, *Curr. Microbiol.*, 16:55-60 (1987)], a 4.7 kb fragment from *PstI* restricted *C. difficile* genomic DNA was gel purified, and ligated to

*Pst*I restricted, phosphatase treated pUC9 DNA. The resulting genomic library was screened with a oligonucleotide primer specific to fragment 3, and multiple independent clones were isolated. The presence of fragment 3 in several of these clones was confirmed by restriction digestion, and a clone of the indicated orientation (Figure 6) was restricted with *Bam*HI/*Hind*III, the released fragment purified by gel electrophoresis, and ligated into similarly restricted pET23c expression vector DNA (Novagen). Recombinant clones were isolated, and confirmed by restriction digestion. This construct is predicted to create both a predicted in frame fusion with the pET protein leader sequence, as well as a predicted C-terminal poly-histidine affinity tag, and is designated pPA1100-2680 (see Figure 6 for the clone designation).

b) Expression Of Large Fragments Of Toxin A In *E. coli*

Protein expression from the three expression constructs made in (a) was induced, and analyzed by Western blot analysis with an affinity purified, goat polyclonal antiserum directed against the toxin A toxoid (Tech Lab). The procedures utilized for protein induction, SDS-PAGE, and Western blot analysis are described in detail in Williams *et al* (1995), *supra*. In brief, 5 ml 2X YT (16 g tryptone, 10 g yeast extract, 5 g NaCl per liter, pH 7.5 + 100 µg/ml ampicillin were added to cultures of bacteria (BL21 for pMA1 and pGEX plasmids, and BL21(DE3)LysS for pET plasmids) containing the appropriate recombinant clone which were induced to express recombinant protein by addition of IPTG to 1 mM. Cultures were grown at 37°C, and induced when the cell density reached 0.5 OD₆₀₀. Induced protein was allowed to accumulate for two hrs after induction. Protein samples were prepared by pelleting 1 ml aliquots of bacteria by centrifugation (1 min in a microfuge), and resuspension of the pelleted bacteria in 150 µl of 2x SDS-PAGE sample buffer [Williams *et al.* (1995), *supra*]. The samples were heated to 95°C for 5 min, the cooled and 5 or 10 µl aliquots loaded on 7.5% SDS-PAGE gels. BioRad high molecular weight protein markers were also loaded, to allow estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining gels with Coomassie

blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein. Western blots, (performed as described in Example 3) which detect toxin A reactive protein in cell lysates of induced protein from the three expression constructs are shown in Figure 7. In this figure, lanes 1-3 contain cell lysates prepared from *E. coli* strains containing pPA1100-2860 in B121(DE3)lysE cells; lanes 4-6 contain cell lysates prepared from *E. coli* strains containing pPA1100-2860 in B121(DE3)lysS cells; lanes 7-9 contain cell lysates prepared from *E. coli* strains containing pMA30-660; lanes 10-12 contain cell lysates prepared from *E. coli* strains containing pMA660-1100. The lanes were probed with an affinity purified goat antitoxin A polyclonal antibody (Tech Lab). Control lysates from uninduced cells (lanes 1, 7, and 10) contain very little immunoreactive material compared to the induced samples in the remaining lanes. The highest molecular weight band observed for each clone is consistent with the predicted size of the full length fusion protein.

Each construct directs expression of high molecular weight (HMW) protein that is reactive with the toxin A antibody. The size of the largest immunoreactive bands from each sample is consistent with predictions of the estimated MW of the intact fusion proteins. This demonstrates that the three fusions are in-frame, and that none of the clones contain cloning artifacts that disrupt the integrity of the encoded fusion protein. However, the Western blot demonstrates that fusion protein from the two larger constructs (pGA30-660 and pPA1100-2680) are highly degraded. Also, expression levels of toxin A proteins from these two constructs are low, since induced protein bands are not visible by Coomassie staining (not shown). Several other expression constructs that fuse large sub-regions of the toxin A gene to either pMALc or pET23a-c expression vectors, were constructed and tested for protein induction. These constructs were made by mixing gel purified restriction fragments, derived from the expression constructs shown in Figure 6, with appropriately cleaved expression vectors, ligating, and selecting recombinant clones in which the toxin A restriction fragments had ligated together and into the expression vector as predicted for in-frame fusions. The expressed toxin A interval within these constructs are shown in Figure 8, as well as the internal restriction sites utilized to make these constructs.

As used herein, the term "interval" refers to any portion (*i.e.*, any segment of the toxin which is less than the whole toxin molecule) of a clostridial toxin. In a preferred embodiment, "interval" refers to portions of *C. difficile* toxins such as toxin A or toxin B. It is also contemplated that these intervals will correspond to epitopes of immunologic importance, such as antigens or immunogens against which a neutralizing antibody response is effected. It is not intended that the present invention be limited to the particular intervals or sequences described in these Examples. It is also contemplated that sub-portions of intervals (*e.g.*, an epitope contained within one interval or which bridges multiple intervals) be used as compositions and in the methods of the present invention.

In all cases, Western blot analysis of each of these constructs with goat antitoxin A antibody (Tech Lab) detected HMW fusion protein of the predicted size (not shown). This confirms that the reading frame of each of these clones is not prematurely terminated, and is fused in the correct frame with the fusion partner. However, the Western blot analysis revealed that in all cases, the induced protein is highly degraded, and, as assessed by the absence of identifiable induced protein bands by Coomassie Blue staining, are expressed only at low levels. These results suggest that expression of high levels of intact toxin A recombinant protein is not possible when large regions of the toxin A gene are expressed in *E. coli* using these expression vectors.

**c) High Level Expression Of Small Toxin A Protein
Fusions In *E. coli***

Experience indicates that expression difficulties are often encountered when large (greater than 100 kd) fragments are expressed in *E. coli*. A number of expression constructs containing smaller fragments of the toxin A gene were constructed, to determine if small regions of the gene can be expressed to high levels without extensive protein degradation. A summary of these expression constructs are shown in Figure 9. All were constructed by in-frame fusions of convenient toxin A

restriction fragments to either the pMALc or pET23a-c vectors. Protein preparations from induced cultures of each of these constructs were analyzed by both Coomassie Blue staining and Western analysis as in (b) above. In all cases, higher levels of intact, full length fusion proteins were observed than with the larger recombinants from section (b).

d) Purification Of Recombinant Toxin A Protein

Large scale (500 ml) cultures of each recombinant from (c) were grown, induced, and soluble and insoluble protein fractions were isolated. The soluble protein extracts were affinity chromatographed to isolate recombinant fusion protein, as described [Williams *et al.* (1994), *supra*]. In brief, extracts containing tagged pET fusions were chromatographed on a nickel chelate column, and eluted using imidazole salts as described by the distributor (Novagen). Extracts containing soluble pMAL fusion protein were prepared and chromatographed in column buffer (10 mM NaPO₄, 0.5M NaCl, 10 mM β-mercaptoethanol, pH 7.2) over an amylose resin column (New England Biolabs), and eluted with column buffer containing 10 mM maltose as described [Williams *et al.* (1995), *supra*]. When the expressed protein was found to be predominantly insoluble, insoluble protein extracts were prepared by the method described in Example 17, *infra*. The results are summarized in Table 16. Figure 10 shows the sample purifications of recombinant toxin A protein. In this figure, lanes 1 and 2 contain MBP fusion protein purified by affinity purification of soluble protein.

TABLE 16

Purification Of Recombinant Toxin A Protein

Clone ^(a)	Protein Solubility	Yield Affinity Purified Soluble Protein ^(b)	% Intact Soluble Fusion Protein ^(c)	Yield Intact Insoluble Fusion Protein
pMA30-270	Soluble	4 mg/500 mls	10%	NA
PMA30-300	Soluble	4 mg/500 mls	5-10%	NA
pMA300-660	Insoluble	—	NA	10 mg/500 ml
pMA660-1100	Soluble	4.5 mg/500 mls	50%	NA
pMA1100-1610	Soluble	18 mg/500 mls	10%	NA
pMA1610-1870	Both	22 mg/500 mls	90%	20 mg/500 ml
pMA1450-1870	Insoluble	-----	NA	0.2 mg/500 ml
pPA1100-1450	Soluble	0.1 mg/500 mls	90%	NA
pPA1100-1870	Soluble	0.02 mg/500 mls	90%	NA
pMA1870-2680	Both	12 mg/500 mls	80%	NA
pPa1870-2680	Insoluble	-----	NA	10 mg/500 ml

^(a) pP = pET23 vector, pM=pMALc vector, A=toxin A.

^(b) Based on 1.5 OD₂₈₀ = 1 mg/ml (extinction coefficient of MBP).

^(c) Estimated by Coomassie staining of SDS-PAGE gels.

Lanes 3 and 4 contain MBP fusion protein purified by solubilization of insoluble inclusion bodies. The purified fusion protein samples are pMA1870-2680 (lane 1), pMA660-1100 (lane 2), pMA300-600 (lane 3) and pMA1450-1870 (lane 4).

Poor yields of affinity purified protein were obtained when poly-histidine tagged pET vectors were used to drive expression (pPA1100-1450, pP1100-1870). However, significant protein yields were obtained from pMAL expression constructs spanning the entire toxin A gene, and yields of full-length soluble fusion protein ranged from an estimated 200-400 µg/500 ml culture (pMA30-300) to greater than 20 mg/500 ml culture (pMA1610-1870). Only one interval was expressed to high levels as strictly insoluble protein (pMA300-660). Thus, although high level expression was not observed when using large expression constructs from the toxin A gene, usable levels of recombinant protein spanning the entire toxin A gene were obtainable by

isolating induced protein from a series of smaller pMAL expression constructs that span the entire toxin A gene. This is the first demonstration of the feasibility of expressing recombinant toxin A protein to high levels in *E. coli*.

e) **Hemagglutination Assay Using The Toxin A
Recombinant Proteins**

The carboxy terminal end consisting of the repeating units contains the hemagglutination activity or binding domain of *C. difficile* toxin A. To determine whether the expressed toxin A recombinants retain functional activity, hemagglutination assays were performed. Two toxin A recombinant proteins, one containing the binding domain as either soluble affinity purified protein (pMA1870-2680) or SDS solubilized inclusion body protein (pPA1870-2680) and soluble protein from one region outside that domain (pMA1100-1610) were tested using a described procedure. [H.C. Krivan *et al.*, Infect. Immun., 53:573 (1986).] Citrated rabbit red blood cells (RRBC)(Cocalico) were washed several times with Tris-buffer (0.1M Tris and 50 mM NaCl) by centrifugation at 450 x g for 10 minutes at 4° C. A 1% RRBC suspension was made from the packed cells and resuspended in Tris-buffer. Dilutions of the recombinant proteins and native toxin A (Tech Labs) were made in the Tris-buffer and added in duplicate to a round-bottomed 96-well microtiter plate in a final volume of 100 µl. To each well, 50 µl of the 1% RRBC suspension was added, mixed by gentle tapping, and incubated at 4°C for 3-4 hours. Significant hemagglutination occurred only in the recombinant proteins containing the binding domain (pMA 1870-2680) and native toxin A. The recombinant protein outside the binding domain (pMA 1100-1610) displayed no hemagglutination activity. Using equivalent protein concentrations, the hemagglutination titer for toxin A was 1:256, while titers for the soluble and insoluble recombinant proteins of the binding domain were 1:256 and about 1:5000. Clearly, the recombinant proteins tested retained functional activity and were able to bind RRBC's.

EXAMPLE 12

Functional Activity Of IgY Reactive Against Toxin A Recombinants

The expression of recombinant toxin A protein as multiple fragments in *E.coli* has demonstrated the feasibility of generating toxin A antigen through use of recombinant methodologies (Example 11). The isolation of these recombinant proteins allows the immunoreactivity of each individual subregion of the toxin A protein to be determined (*i.e.*, in a antibody pool directed against the native toxin A protein). This identifies the regions (if any) for which little or no antibody response is elicited when the whole protein is used as a immunogen. Antibodies directed against specific fragments of the toxin A protein can be purified by affinity chromatography against recombinant toxin A protein, and tested for neutralization ability. This identifies any toxin A subregions that are essential for producing neutralizing antibodies. Comparison with the levels of immune response directed against these intervals when native toxin is used as an immunogen predicts whether potentially higher titers of neutralizing antibodies can be produced by using recombinant protein directed against a individual region, rather than the entire protein. Finally, since it is unknown whether antibodies reactive to the recombinant toxin A proteins produced in Example 11 neutralize toxin A as effectively as antibodies raised against native toxin A (Examples 9 and 10), the protective ability of a pool of antibodies affinity purified against recombinant toxin A fragments was assessed for its ability to neutralize toxin A.

This Example involved (a) epitope mapping of the toxin A protein to determine the titre of specific antibodies directed against individual subregions of the toxin A protein when native toxin A protein is used as an immunogen, (b) affinity purification of IgY reactive against recombinant proteins spanning the toxin A gene, (c) toxin A neutralization assays with affinity purified IgY reactive to recombinant toxin A protein to identify subregions of the toxin A protein that induce the production of neutralizing antibodies, and determination of whether complete neutralization of toxin A can be elicited with a mixture of antibodies reactive to recombinant toxin A protein.

a) Epitope Mapping Of The Toxin A Gene

The affinity purification of recombinant toxin A protein specific to defined intervals of the toxin A protein allows epitope mapping of antibody pools directed against native toxin A. This has not previously been possible, since previous
5 expression of toxin A recombinants has been assessed only by Western blot analysis, without knowledge of the expression levels of the protein [e.g., von Eichel-Streiber *et al.*, J. Gen. Microbiol., 135:55-64 (1989)]. Thus, high or low reactivity of recombinant toxin A protein on Western blots may reflect protein expression level differences, not
10 immunoreactivity differences. Given that the purified recombinant protein generated in Example 11 have been quantitated, the issue of relative immunoreactivity of individual regions of the toxin A protein was precisely addressed.

For the purposes of this Example, the toxin A protein was subdivided into 6 intervals (1-6), numbered from the amino (interval 1) to the carboxyl (interval 6) termini.

15 The recombinant proteins corresponding to these intervals were from expression clones (see Example 11(d) for clone designations) pMA30-300 (interval 1), pMA300-660 (interval 2), pMA660-1100 (interval 3), pPA1100-1450 (interval 4), pMA1450-1870 (interval 5) and pMA1870-2680 (interval 6). These 6 clones were selected because they span the entire protein from amino acids numbered 30 through 2680, and
20 subdivide the protein into 6 small intervals. Also, the carbohydrate binding repeat interval is contained specifically in one interval (interval 6), allowing evaluation of the immune response specifically directed against this region. Western blots of 7.5% SDS-PAGE gels, loaded and electrophoresed with defined quantities of each recombinant protein, were probed with either goat antitoxin A polyclonal antibody
25 (Tech Lab) or chicken antitoxin A polyclonal antibody [pCTA IgY, Example 8(c)]. The blots were prepared and developed with alkaline phosphatase as previously described [Williams *et al.* (1995), *supra*]. At least 90% of all reactivity, in either goat or chicken antibody pools, was found to be directed against the ligand binding domain (interval 6). The remaining immunoreactivity was directed against all five remaining

intervals, and was similar in both antibody pools, except that the chicken antibody showed a much lower reactivity against interval 2 than the goat antibody.

This clearly demonstrates that when native toxin A is used as an immunogen in goats or chickens, the bulk of the immune response is directed against the ligand binding domain of the protein, with the remaining response distributed throughout the remaining 2/3 of the protein.

b) Affinity Purification Of IgY Reactive Against Recombinant Toxin A Protein

Affinity columns, containing recombinant toxin A protein from the 6 defined intervals in (a) above, were made and used to (i) affinity purify antibodies reactive to each individual interval from the CTA IgY preparation [Example 8(c)], and (ii) deplete interval specific antibodies from the CTA IgY preparation. Affinity columns were made by coupling 1 ml of PBS-washed Actigel resin (Sterogene) with region specific protein and 1/10 final volume of Ald-coupling solution (1M sodium cyanoborohydride). The total region specific protein added to each reaction mixture was 2.7 mg (interval 1), 3 mg (intervals 2 and 3), 0.1 mg (interval 4), 0.2 mg (interval 5) and 4 mg (interval 6). Protein for intervals 1, 3, and 6 was affinity purified pMAL fusion protein in column buffer (see Example 11). Interval 4 was affinity purified poly-histidine containing pET fusion in PBS; intervals 2 and 5 were from inclusion body preparations of insoluble pMAL fusion protein, dialyzed extensively in PBS. Aliquots of the supernatants from the coupling reactions, before and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based on protein band intensities, in all cases greater than 50% coupling efficiencies were estimated. The resins were poured into 5 ml BioRad columns, washed extensively with PBS, and stored at 4°C.

Aliquots of the CTA IgY polyclonal antibody preparation were depleted for each individual region as described below. A 20 ml sample of the CTA IgY preparation [Example 8(c)] was dialyzed extensively against 3 changes of PBS (1 liter for each dialysis), quantitated by absorbance at OD₂₈₀, and stored at 4°C. Six 1 ml

aliquots of the dialyzed IgY preparation were removed, and depleted individually for each of the six intervals. Each 1 ml aliquot was passed over the appropriate affinity column, and the eluate twice reapplied to the column. The eluate was collected, and pooled with a 1 ml PBS wash. Bound antibody was eluted from the column by washing with 5 column volumes of 4 M Guanidine-HCl (in 10 mM Tris-HCl, pH 8.0). The column was reequilibrated in PBS, and the depleted antibody stock reapplied as described above. The eluate was collected, pooled with a 1 ml PBS wash, quantitated by absorbance at OD₂₈₀, and stored at 4° C. In this manner, 6 aliquots of the CTA IgY preparation were individually depleted for each of the 6 toxin A intervals, by two rounds of affinity depletion. The specificity of each depleted stock was tested by Western blot analysis. Multiple 7.5% SDS-PAGE gels were loaded with protein samples corresponding to all 6 toxin A subregions. After electrophoresis, the gels were blotted, and protein transfer confirmed by Ponceau S staining [protocols described in Williams *et al.* (1995), *supra*]. After blocking the blots 1 hr at 20°C in PBS+ 0.1% Tween 20 (PBST) containing 5% milk (as a blocking buffer), 4 ml of either a 1/500 dilution of the dialyzed CTA IgY preparation in blocking buffer, or an equivalent amount of the six depleted antibody stocks (using OD₂₈₀ to standardize antibody concentration) were added and the blots incubated a further 1 hr at room temperature. The blots were washed and developed with alkaline phosphatase (using a rabbit anti-chicken alkaline phosphate conjugate as a secondary antibody) as previously described [Williams *et al.* (1995), *supra*]. In all cases, only the target interval was depleted for antibody reactivity, and at least 90% of the reactivity to the target intervals was specifically depleted.

Region specific antibody pools were isolated by affinity chromatography as described below. Ten mls of the dialyzed CTA IgY preparation were applied sequentially to each affinity column, such that a single 10 ml aliquot was used to isolate region specific antibodies specific to each of the six subregions. The columns were sequentially washed with 10 volumes of PBS, 6 volumes of BBS-Tween, 10 volumes of TBS, and eluted with 4 ml Actisep elution media (Sterogene). The eluate was dialyzed extensively against several changes of PBS, and the affinity purified

antibody collected and stored at 4°C. The volumes of the eluate increased to greater than 10 mls during dialysis in each case, due to the high viscosity of the Actisep elution media. Aliquots of each sample were 20x concentrated using Centricon 30 microconcentrators (Amicon) and stored at 4°C. The specificity of each region specific antibody pool was tested, relative to the dialyzed CTA IgY preparation, by Western blot analysis, exactly as described above, except that 4 ml samples of blocking buffer containing 100 µl region specific antibody (unconcentrated) were used instead of the depleted CTA IgY preparations. Each affinity purified antibody preparation was specific to the defined interval, except that samples purified against intervals 1-5 also reacted with interval 6. This may be due to non-specific binding to the interval 6 protein, since this protein contains the repetitive ligand binding domain which has been shown to bind antibodies nonspecifically. [Lyerly *et al.*, Curr. Microbiol., 19:303-306 (1989).]

The reactivity of each affinity purified antibody preparation to the corresponding proteins was approximately the same as the reactivity of the 1/500 diluted dialyzed CTA IgY preparation standard. Given that the specific antibody stocks were diluted 1/40, this would indicate that the unconcentrated affinity purified antibody stocks contain 1/10-1/20 the concentration of specific antibodies relative to the starting CTA IgY preparation.

**c) Toxin A Neutralization Assay Using Antibodies
Reactive Toward Recombinant Toxin A Protein**

The CHO toxin neutralization assay [Example 8(d)] was used to assess the ability of the depleted or enriched samples generated in (b) above to neutralize the cytotoxicity of toxin A. The general ability of affinity purified antibodies to neutralize toxin A was assessed by mixing together aliquots of all 6 concentrated stocks of the 6 affinity purified samples generated in (b) above, and testing the ability of this mixture to neutralize a toxin A concentration of 0.1 µg/ml. The results, shown in Figure 11, demonstrate almost complete neutralization of toxin A using the affinity purified (AP) mix. Some epitopes within the recombinant proteins utilized for affinity purification

were probably lost when the proteins were denatured before affinity purification [by Guanidine-HCl treatment in (b) above]. Thus, the neutralization ability of antibodies directed against recombinant protein is probably underestimated using these affinity purified antibody pools. This experiment demonstrates that antibodies reactive to recombinant toxin A can neutralize cytotoxicity, suggesting that neutralizing antibodies may be generated by using recombinant toxin A protein as immunogen.

In view of the observation that the recombinant expression clones of the toxin A gene divide the protein into 6 subregions, the neutralizing ability of antibodies directed against each individual region was assessed. The neutralizing ability of antibodies directed against the ligand binding domain of toxin A was determined first.

In the toxin neutralization experiment shown in Figure 11, interval 6 specific antibodies (interval 6 contains the ligand binding domain) were depleted from the dialyzed PEG preparation, and the effect on toxin neutralization assayed. Interval 6 antibodies were depleted either by utilizing the interval 6 depleted CTA IgY preparation from (b) above ("-6 aff. depleted" in Figure 11), or by addition of interval 6 protein to the CTA IgY preparation (estimated to be a 10 fold molar excess over anti-interval 6 immunoglobulin present in this preparation) to competitively compete for interval 6 protein ("-6 prot depleted" in Figure 11). In both instances, removal of interval 6 specific antibodies reduces the neutralization efficiency relative to the starting CTA IgY preparation. This demonstrates that antibodies directed against interval 6 contribute to toxin neutralization. Since interval 6 corresponds to the ligand binding domain of the protein, these results demonstrate that antibodies directed against this region in the PEG preparation contribute to the neutralization of toxin A in this assay. However, it is significant that after removal of these antibodies, the PEG preparation retains significant ability to neutralize toxin A (Figure 11). This neutralization is probably due to the action of antibodies specific to other regions of the toxin A protein, since at least 90% of the ligand binding region reactive antibodies were removed in the depleted sample prepared in (b) above. This conclusion was supported by comparison of the toxin neutralization of the affinity purified (AP) mix compared to affinity purified interval 6 antibody alone. Although some neutralization

ability was observed with AP interval 6 antibodies alone, the neutralization was significantly less than that observed with the mixture of all 6 AP antibody stocks (not shown).

Given that the mix of all six affinity purified samples almost completely neutralized the cytotoxicity of toxin A (Figure 11), the relative importance of antibodies directed against toxin A intervals 1-5 within the mixture was determined. This was assessed in two ways. First, samples containing affinity purified antibodies representing 5 of the 6 intervals were prepared, such that each individual region was depleted from one sample. Figure 12 demonstrates a sample neutralization curve, comparing the neutralization ability of affinity purified antibody mixes without interval 4 (-4) or 5 (-5) specific antibodies, relative to the mix of all 6 affinity purified antibody stocks (positive control). While the removal of interval 5 specific antibodies had no effect on toxin neutralization (or intervals 1-3, not shown), the loss of interval 4 specific antibodies significantly reduced toxin neutralization (Figure 12).

Similar results were seen in a second experiment, in which affinity purified antibodies, directed against a single region, were added to interval 6 specific antibodies, and the effects on toxin neutralization assessed. Only interval 4 specific antibodies significantly enhanced neutralization when added to interval 6 specific antibodies (Figure 13). These results demonstrate that antibodies directed against interval 4 (corresponding to clone pPA1100-1450 in Figure 9) are important for neutralization of cytotoxicity in this assay. Epitope mapping has shown that only low levels of antibodies reactive to this region are generated when native toxin A is used as an immunogen [Example 12(a)]. It is hypothesized that immunization with recombinant protein specific to this interval will elicit higher titers of neutralizing antibodies. In summary, this analysis has identified two critical regions of the toxin A protein against which neutralizing antibodies are produced, as assayed by the CHO neutralization assay.

EXAMPLE 13

Production And Evaluation Of Avian Antitoxin Against *C. difficile* Recombinant Toxin A Polypeptide

In Example 12, we demonstrated neutralization of toxin A mediated
5 cytotoxicity by affinity purified antibodies reactive to recombinant toxin A protein. To
determine whether antibodies raised against a recombinant polypeptide fragment of
C. difficile toxin A may be effective in treating clostridial diseases, antibodies to
recombinant toxin A protein representing the binding domain were generated. Two
toxin A binding domain recombinant polypeptides, expressing the binding domain in
10 either the pMALc (pMA1870-2680) or pET 23(pPA1870-2680) vector, were used as
immunogens. The pMAL protein was affinity purified as a soluble product
[Example 12(d)] and the pET protein was isolated as insoluble inclusion bodies
[Example 12(d)] and solubilized to an immunologically active protein using a
proprietary method described in a pending patent application (U.S. Patent Application
15 Serial No. 08/129,027). This Example involves (a) immunization, (b) antitoxin
collection, (c) determination of antitoxin antibody titer, (d) anti-recombinant toxin A
neutralization of toxin A hemagglutination activity *in vitro*, and (e) assay of *in vitro*
toxin A neutralizing activity.

a) Immunization

20 The soluble and the inclusion body preparations each were used separately to
immunize hens. Both purified toxin A polypeptides were diluted in PBS and
emulsified with approximately equal volumes of CFA for the initial immunization or
IFA for subsequent booster immunizations. On day zero, for each of the recombinant
preparations, two egg laying white Leghorn hens (obtained from local breeder) were
25 each injected at multiple sites (intramuscular and subcutaneous) with 1 ml of
recombinant adjuvant mixture containing approximately 0.5 to 1.5 mgs of recombinant
toxin A. Booster immunizations of 1.0 mg were given on days 14 and day 28.

b) Antitoxin Collection

Total yolk immune IgY was extracted as described in the standard PEG protocol (as in Example 1) and the final IgY pellet was dissolved in sterile PBS at the original yolk volume. This material is designated "immune recombinant IgY" or
5 "immune IgY."

c) Antitoxin Antibody Titer

To determine if the recombinant toxin A protein was sufficiently immunogenic to raise antibodies in hens, the antibody titer of a recombinant toxin A polypeptide was determined by ELISA. Eggs from both hens were collected on day 32, the yolks
10 pooled and the antibody was isolated using PEG as described. The immune recombinant IgY antibody titer was determined for the soluble recombinant protein containing the maltose binding protein fusion generated in p-Mal (pMA1870-2680). Ninety-six well Falcon Pro-bind plates were coated overnight at 4°C with 100 µl /well of toxin A recombinant at 2.5 µg /µl in PBS containing 0.05% thimerosal. Another
15 plate was also coated with maltose binding protein (MBP) at the same concentration, to permit comparison of antibody reactivity to the fusion partner. The next day, the wells were blocked with PBS containing 1% bovine serum albumin (BSA) for 1 hour at 37°C. IgY isolated from immune or preimmune eggs was diluted in antibody diluent (PBS containing 1% BSA and 0.05% Tween-20), and added to the blocked
20 wells and incubated for 1 hour at 37°C. The plates were washed three times with PBS with 0.05% Tween-20, then three times with PBS. Alkaline phosphatase conjugated rabbit anti-chicken IgG (Sigma) diluted 1:1000 in antibody diluent was added to the plate, and incubated for 1 hour at 37°C. The plates were washed as before and substrate was added, [p-nitrophenyl phosphate (Sigma)] at 1 mg/ml in
25 0.05M Na₂CO₃, pH 9.5 and 10 mM MgCl₂. The plates were evaluated quantitatively on a Dynatech MR 300 Micro EPA plate reader at 410 nm about 10 minutes after the addition of substrate.

Based on these ELISA results, high antibody titers were raised in chickens immunized with the toxin A recombinant polypeptide. The recombinant appeared to

be highly immunogenic, as it was able to generate high antibody titers relatively quickly with few immunizations. Immune IgY titer directed specifically to the toxin A portion of the recombinant was higher than the immune IgY titer to its fusion partner, the maltose binding protein, and significantly higher than the preimmune IgY. ELISA titers (reciprocal of the highest dilution of IgY generating a signal) in the preimmune IgY to the MBP or the recombinant was <1:30 while the immune IgY titers to MBP and the toxin A recombinant were 1:18750 and > 1:93750 respectively. Importantly, the anti-recombinant antibody titers generated in the hens against the recombinant polypeptide is much higher, compared to antibodies to that region raised using native toxin A. The recombinant antibody titer to region 1870-2680 in the CTA antibody preparation is at least five-fold lower compared to the recombinant generated antibodies (1:18750 versus >1:93750). Thus, it appears a better immune response can be generated against a specific recombinant using that recombinant as the immunogen compared to the native toxin A.

This observation is significant, as it shows that because recombinant portions stimulate the production of antibodies, it is not necessary to use native toxin molecules to produce antitoxin preparations. Thus, the problems associated with the toxicity of the native toxin are avoided and large-scale antitoxin production is facilitated.

d) Anti-Recombinant Toxin A Neutralization Of Toxin A Hemagglutination Activity *In Vitro*

Toxin A has hemagglutinating activity besides cytotoxic and enterotoxin properties. Specifically, toxin A agglutinates rabbit erythrocytes by binding to a trisaccharide (gal 1-3B1-4GlcNAc) on the cell surface. [H. Krivan *et al.*, Infect. Immun., 53:573-581 (1986).] We examined whether the anti-recombinant toxin A (immune IgY, antibodies raised against the insoluble product expressed in pET) can neutralize the hemagglutination activity of toxin A in vitro. The hemagglutination assay procedure used was described by H.C. Krivan *et al.* Polyethylene glycol-fractionated immune or preimmune IgY were pre-absorbed with citrated rabbit erythrocytes prior to performing the hemagglutination assay because we have found

that IgY alone can agglutinate red blood cells. Citrated rabbit red blood cells (RRBC's)(Cocalico) were washed twice by centrifugation at 450 x g with isotonic buffer (0.1 M Tris-HCl, 0.05 M NaCl, pH 7.2). RRBC-reactive antibodies in the IgY were removed by preparing a 10% RRBC suspension (made by adding packed cells to immune or preimmune IgY) and incubating the mixture for 1 hour at 37°C. The RRBCs were then removed by centrifugation. Neutralization of the hemagglutination activity of toxin A by antibody was tested in round-bottomed 96-well microtiter plates. Twenty-five µl of toxin A (36 µg /ml) (Tech Lab) in isotonic buffer was mixed with an equal volume of different dilutions of immune or preimmune IgY in isotonic buffer, and incubated for 15 minutes at room temperature. Then, 50 µl of a 1% RRBC suspension in isotonic buffer was added and the mixture was incubated for 3 hours at 4°C. Positive control wells containing the final concentration of 9 µg/ml of toxin A after dilution without IgY were also included. Hemagglutination activity was assessed visually, with a diffuse matrix of RRBC's coating the bottom of the well representing a positive hemagglutination reaction and a tight button of RRBC's at the bottom of the well representing a negative reaction. The anti-recombinant immune IgY neutralized toxin A hemagglutination activity, giving a neutralization titer of 1:8. However, preimmune IgY was unable to neutralize the hemagglutination ability of toxin A.

e) Assay Of *In Vitro* Toxin A Neutralizing Activity

The ability of the anti-recombinant toxin A IgY (immune IgY antibodies raised against pMA1870-2680, the soluble recombinant binding domain protein expressed in pMAL, designated as Anti-tox. A-2 in Figure 14 , and referred to as recombinant region 6) and pre-immune IgY, prepared as described in Example 8(c) above, to neutralize the cytotoxic activity of toxin A was assessed *in vitro* using the CHO cell cytotoxicity assay, and toxin A (Tech Lab) at a concentration of 0.1µg/ml, as described in Example 8(d) above. As additional controls, the anti-native toxin A IgY (CTA) and pre-immune IgY preparations described in Example 8(c) above were also tested. The results are shown in Figure 14.

The anti-recombinant toxin A IgY demonstrated only partial neutralization of the cytotoxic activity of toxin A, while the pre-immune IgY did not demonstrate any significant neutralizing activity.

EXAMPLE 14

In vivo Neutralization Of *C. difficile* Toxin A

The ability of avian antibodies (IgY) raised against recombinant toxin A binding domain to neutralize the enterotoxin activity of *C. difficile* toxin A was evaluated *in vivo* using Golden Syrian hamsters. The Example involved:

- (a) preparation of the avian anti-recombinant toxin A IgY for oral administration;
- (b) *in vivo* protection of hamsters from *C. difficile* toxin A enterotoxicity by treatment of toxin A with avian anti-recombinant toxin A IgY; and (c) histologic evaluation of hamster ceca.

a) Preparation Of The Avian Anti-Recombinant Toxin A IgY For Oral Administration

Eggs were collected from hens which had been immunized with the recombinant *C. difficile* toxin A fragment pMA1870-2680 (described in Example 13, above). A second group of eggs purchased at a local supermarket was used as a pre-immune (negative) control. Egg yolk immunoglobulin (IgY) was extracted by PEG from the two groups of eggs as described in Example 8(c), and the final IgY pellets were solubilized in one-fourth the original yolk volume using 0.1M carbonate buffer (mixture of NaHCO_3 and Na_2CO_3), pH 9.5. The basic carbonate buffer was used in order to protect the toxin A from the acidic pH of the stomach environment.

b) ***In vivo* Protection Of Hamsters Against *C. difficile*
Toxin A Enterotoxicity By Treatment Of Toxin A
With Avian Anti-recombinant Toxin A IgY**

5 In order to assess the ability of the avian anti-recombinant toxin A IgY,
prepared in section (a) above to neutralize the *in vivo* enterotoxin activity of toxin A,
an *in vivo* toxin neutralization model was developed using Golden Syrian hamsters.
This model was based on published values for the minimum amount of toxin A
required to elicit diarrhea (0.08 mg toxin A/Kg body wt.) and death (0.16 mg toxin
A/Kg body wt.) in hamsters when administered orally (Lyerly *et al.* Infect. Immun.,
10 47:349-352 (1985).

For the study, four separate experimental groups were used, with each group
consisting of 7 female Golden Syrian hamsters (Charles River), approx. three and one-
half weeks old, weighing approx. 50 gms each. The animals were housed as groups
of 3 and 4, and were offered food and water *ad libitum* through the entire length of
15 the study.

For each animal, a mixture containing either 10µg of toxin A (0.2 mg/Kg) or
30µg of toxin A (0.6 mg/Kg) (*C. difficile* toxin A was obtained from Tech Lab and
1 ml of either the anti-recombinant toxin A IgY or pre-immune IgY (from section (a)
above) was prepared. These mixtures were incubated at 37°C for 60 min. and were
20 then administered to the animals by the oral route. The animals were then observed
for the onset of diarrhea and death for a period of 24 hrs. following the administration
of the toxin A+IgY mixtures, at the end of which time, the following results were
tabulated and shown in Table 17:

TABLE 17

Study Outcome At 24 Hours

Experimental Group	Study Outcome at 24 Hours		
	Healthy ¹	Diarrhea ²	Dead ³
10 µg Toxin A + Antitoxin Against Interval 6	7	0	0
30 µg Toxin A + Antitoxin Against Interval 6	7	0	0
10 µg Toxin A + Pre-Immune Serum	0	5	2
30 µg Toxin A + Pre-Immune	0	5	2

¹ Animals remained healthy through the entire 24 hour study period.

² Animals developed diarrhea, but did not die.

³ Animals developed diarrhea, and subsequently died.

Pretreatment of toxin A at both doses tested, using the anti-recombinant toxin A IgY, prevented all overt symptoms of disease in hamsters. Therefore, pretreatment of *C. difficile* toxin A, using the anti-recombinant toxin A IgY, neutralized the *in vivo* enterotoxin activity of the toxin A. In contrast, all animals from the two groups which received toxin A which had been pretreated using pre-immune IgY developed disease symptoms which ranged from diarrhea to death. The diarrhea which developed in the 5 animals which did not die in each of the two pre-immune groups, spontaneously resolved by the end of the 24 hr. study period.

c) Histologic Evaluation Of Hamster Ceca

In order to further assess the ability of anti-recombinant toxin A IgY to protect hamsters from the enterotoxin activity of toxin A, histologic evaluations were performed on the ceca of hamsters from the study described in section (b) above.

Three groups of animals were sacrificed in order to prepare histological specimens. The first group consisted of a single representative animal taken from each of the 4 groups of surviving hamsters at the conclusion of the study described in section (b) above. These animals represented the 24 hr. timepoint of the study.

The second group consisted of two animals which were not part of the study described above, but were separately treated with the same toxin A + pre-immune IgY

mixtures as described for the animals in section (b) above. Both of these hamsters developed diarrhea, and were sacrificed 8 hrs. after the time of administration of the toxin A + pre-immune IgY mixtures. At the time of sacrifice, both animals were presenting symptoms of diarrhea. These animals represented the acute phase of the study.

The final group consisted of a single untreated hamster from the same shipment of animals as those used for the two previous groups. This animal served as the normal control.

Samples of cecal tissue were removed from the 7 animals described above, and were fixed overnight at 4°C using 10% buffered formalin. The fixed tissues were paraffin-embedded, sectioned, and mounted on glass microscope slides. The tissue sections were then stained using hematoxylin and eosin (H and E stain), and were examined by light microscopy.

The tissues obtained from the two 24 hr. animals which received mixtures containing either 10µg or 30µg of toxin A and anti-recombinant toxin A IgY were indistinguishable from the normal control, both in terms of gross pathology, as well as at the microscopic level. These observations provide further evidence for the ability of anti-recombinant toxin A IgY to effectively neutralize the *in vivo* enterotoxin activity of *C. difficile* toxin A, and thus its ability to prevent acute or lasting toxin A-induced pathology.

In contrast, the tissues from the two 24 hr. animals which received the toxin A + pre-immune IgY mixtures demonstrated significant pathology. In both of these groups, the mucosal layer was observed to be less organized than in the normal control tissue. The cytoplasm of the epithelial cells had a vacuolated appearance, and gaps were present between the epithelium and the underlying cell layers. The lamina propria was largely absent. Intestinal villi and crypts were significantly diminished, and appeared to have been overgrown by a planar layer of epithelial cells and fibroblasts. Therefore, although these animals overtly appeared to recover from the acute symptoms of toxin A intoxication, lasting pathologic alterations to the cecal mucosa had occurred.

The tissues obtained from the two acute animals which received mixtures of toxin A and pre-immune IgY demonstrated the most significant pathology. At the gross pathological level, both animals were observed to have severely distended ceca which were filled with watery, diarrhea-like material. At the microscopic level, the animal that was given the mixture containing 10µg of toxin A and pre-immune IgY was found to have a mucosal layer which had a ragged, damaged appearance, and a disorganized, compacted quality. The crypts were largely absent, and numerous breaks in the epithelium had occurred. There was also an influx of erythrocytes into spaces between the epithelial layer and the underlying tissue. The animal which had received the mixture containing 30µg of toxin A and pre-immune IgY demonstrated the most severe pathology. The cecal tissue of this animal had an appearance very similar to that observed in animals which had died from *C. difficile* disease. Widespread destruction of the mucosa was noted, and the epithelial layer had sloughed. Hemorrhagic areas containing large numbers of erythrocytes were very prevalent. All semblance of normal tissue architecture was absent from this specimen. In terms of the presentation of pathologic events, this *in vivo* hamster model of toxin A-intoxication correlates very closely with the pathologic consequences of *C. difficile* disease in hamsters. The results presented in this Example demonstrate that while anti-recombinant toxin A (Interval 6) IgY is capable of only partially neutralizing the cytotoxic activity of *C. difficile* toxin A, the same antibody effectively neutralizes 100% of the *in vivo* enterotoxin activity of the toxin. While it is not intended that this invention be limited to this mechanism, this may be due to the cytotoxicity and enterotoxicity of *C. difficile* Toxin A as two separate and distinct biological functions.

EXAMPLE 15

In Vivo Neutralization Of *C. Difficile* Toxin A By Antibodies Against Recombinant Toxin A Polypeptides

The ability of avian antibodies directed against the recombinant *C. difficile* toxin A fragment 1870-2680 (as expressed by pMA1870-2680; see Example 13) to neutralize the enterotoxic activity of toxin A was demonstrated in Example 14. The ability of avian antibodies (IgYs) directed against other recombinant toxin A epitopes to neutralize native toxin A *in vivo* was next evaluated. This example involved: (a) the preparation of IgYs against recombinant toxin A polypeptides; (b) *in vivo* protection of hamsters against toxin A by treatment with anti-recombinant toxin A IgYs and (c) quantification of specific antibody concentration in CTA and Interval 6 IgY PEG preparations.

The nucleotide sequence of the coding region of the entire toxin A protein is listed in SEQ ID NO:5. The amino acid sequence of the entire toxin A protein is listed in SEQ ID NO:6. The amino acid sequence consisting of amino acid residues 1870 through 2680 of toxin A is listed in SEQ ID NO:7. The amino acid sequence consisting of amino acid residues 1870 through 1960 of toxin A is listed in SEQ ID NO:8.

a) Preparation Of IgY's Against Recombinant Toxin A Polypeptides

Eggs were collected from Leghorn hens which have been immunized with recombinant *C. difficile* toxin A polypeptide fragments encompassing the entire toxin A protein. The polypeptide fragments used as immunogens were: 1) pMA 1870-2680 (Interval 6), 2) pPA 1100-1450 (Interval 4), and 3) a mixture of fragments consisting of pMA 30-300 (Interval 1), pMA 300-660 (Interval 2), pMA 660-1100 (Interval 3) and pMA 1450-1870 (Interval 5). This mixture of immunogens is referred to as Interval 1235. The location of each interval within the toxin A molecule is shown in

Figure 15A. In Figure 15A, the following abbreviations are used: pP refers to the pET23 vector (New England BioLabs); pM refers to the pMALTM-c vector (New England BioLabs); A refers to toxin A; the numbers refer to the amino acid interval expressed in the clone. (For example, the designation pMA30-300 indicates that the recombinant clone encodes amino acids 30-300 of toxin A and the vector used was pMALTM-c).

The recombinant proteins were generated as described in Example 11. The IgYs were extracted and solubilized in 0.1M carbonate buffer pH 9.5 for oral administration as described in Example 14(a). The IgY reactivities against each individual recombinant interval was evaluated by ELISA as described in Example 13(c).

**b) In Vivo Protection Of Hamsters Against Toxin
A By Treatment With Anti-Recombinant Toxin
A Antibodies**

The ability of antibodies raised against recombinant toxin A polypeptides to provide *in vivo* protection against the enterotoxic activity of toxin A was examined in the hamster model system. This assay was performed as described in Example 14(b). Briefly, for each 40-50 gram female Golden Syrian hamster (Charles River), 1 ml of IgY 4X (*i.e.*, resuspended in 1/4 of the original yolk volume) PEG prep against Interval 6, Interval 4 or Interval 1235 was mixed with 30 µg (LD₁₀₀ oral dose) of *C. difficile* toxin A (Tech Lab). Preimmune IgY mixed with toxin A served as a negative control. Antibodies raised against *C. difficile* toxoid A (Example 8) mixed with toxin A (CTA) served as a positive control. The mixture was incubated for 1 hour at 37°C then orally administered to lightly etherized hamsters using an 18G feeding needle. The animals were then observed for the onset of diarrhea and death for a period of approximately 24 hours. The results are shown in Table 18.

TABLE 18

Study Outcome After 24 Hours

Treatment group	Healthy ¹	Diarrhea ²	Dead ³
Preimmune	0	0	7
CTA	5	0	0
Interval 6	6	1	0
Interval 4	0	1	6
Interval 1235	0	0	7

¹ Animal shows no sign of illness.

² Animal developed diarrhea, but did not die.

³ Animal developed diarrhea and died.

Pre-treatment of toxin A with IgYs against Interval 6 prevented diarrhea in 6 of 7 hamsters and completely prevented death in all 7. In contrast, as with preimmune IgY, IgYs against Interval 4 and Interval 1235 had no effect on the onset of diarrhea and death in the hamsters.

c) Quantification Of Specific Antibody Concentration In CTA And Interval 6 IgY PEG Preparations

To determine the purity of IgY PEG preparations, an aliquot of a pMA1870-2680 (Interval 6) IgY PEG preparation was chromatographed using HPLC and a KW-803 sizing column (Shodex). The resulting profile of absorbance at 280 nm is shown in Figure 16. The single large peak corresponds to the predicted MW of IgY. Integration of the area under the single large peak showed that greater than 95% of the protein eluted from the column was present in this single peak. This result demonstrated that the majority (>95%) of the material absorbing at 280 nm in the PEG preparation corresponds to IgY. Therefore, absorbance at 280 nm can be used to determine the total antibody concentration in PEG preparations.

To determine the concentration of Interval 6-specific antibodies (expressed as percent of total antibody) within the CTA and pMA1870-2680 (Interval 6) PEG preparations, defined quantities of these antibody preparations were affinity purified on

a pPA1870-2680(H) (shown schematically in Figure 15B) affinity column and the specific antibodies were quantified. In Figure 15B the following abbreviations are used: pP refers to the pET23 vector (New England BioLabs); pM refers to the pMALTM-c vector (New England BioLabs); pG refers to the pGEX vector (Pharmacia); pB refers to the PinPointTM Xa vector (Promega); A refers to toxin A; the numbers refer to the amino acid interval expressed in the clone. The solid black ovals represent the MBP; the hatched ovals represent glutathione S-transferase; the hatched circles represent the biotin tag; and HHH represents the poly-histidine tag.

An affinity column containing recombinant toxin A repeat protein was made as follows. Four ml of PBS-washed Actigel resin (Sterogene) was coupled with 5-10 mg of pPA1870-2680 inclusion body protein [prepared as described in Example (17) and dialyzed into PBS] in a 15 ml tube (Falcon) containing 1/10 final volume Ald-coupling solution (1 M sodium cyanoborohydride). Aliquots of the supernatant from the coupling reactions, before and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based upon protein band intensities, greater than 6 mg of recombinant protein was coupled to the resin. The resin was poured into a 10 ml column (BioRad), washed extensively with PBS, pre-eluted with 4 M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0; 0.005% thimerosal) and re-equilibrated with PBS. The column was stored at 4°C.

Aliquots of a pMA1870-2680 (Interval 6) or a CTA IgY polyclonal antibody preparation (PEG prep) were affinity purified on the above affinity column as follows. The column was attached to an UV monitor (ISCO) and washed with PBS. For pMA1870-2680 IgY purification, a 2X PEG prep (filter sterilized using a 0.45 μ filter; approximately 500 mg total IgY) was applied. The column was washed with PBS until the baseline was re-established (the column flow-through was saved), washed with BBSTween to elute nonspecifically binding antibodies and re-equilibrated with PBS. Bound antibody was eluted from the column in 4 M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0; 0.005% thimerosal). The entire elution peak was collected in a 15 ml tube (Falcon). The column was re-equilibrated and the column eluate was re-chromatographed as described above. The antibody preparation was quantified by UV

absorbance (the elution buffer was used to zero the spectrophotometer). Total purified antibody was approximately 9 mg and 1 mg from the first and second chromatography passes, respectively. The low yield from the second pass indicated that most specific antibodies were removed by the first round of chromatography. The estimated percentage of Interval 6 specific antibodies in the pMA1870-2680 PEG prep is approximately 2%.

The percentage of Interval 6 specific antibodies in the CTA PEG prep was determined (utilizing the same column and methodology described above) to be approximately 0.5% of total IgY.

A 4X PEG prep contains approximately 20 mg/ml IgY. Thus in b) above, approximately 400 μ g specific antibody in the Interval 6 PEG prep neutralized 30 μ g toxin A *in vivo*.

EXAMPLE 16

In Vivo Treatment Of *C. difficile* Disease In Hamsters By Recombinant Interval 6 Antibodies

The ability of antibodies directed against recombinant Interval 6 of toxin A to protect hamsters *in vivo* from *C. difficile* disease was examined. This example involved: (a) prophylactic treatment of *C. difficile* disease and (b) therapeutic treatment of *C. difficile* disease.

a) Prophylactic Treatment Of *C. difficile* Disease

This experiment was performed as described in Example 9(b). Three groups each consisting of 7 female 100 gram Syrian hamsters (Charles River) were prophylactically treated with either preimmune IgYs, IgYs against native toxin A and B [CTAB; see Example 8 (a) and (b)] or IgYs against Interval 6. IgYs were prepared as 4X PEG preparations as described in Example 9(a).

The animals were orally dosed 3 times daily, roughly at 4 hour intervals, for 12 days with 1 ml antibody preparations diluted in Ensure®. Using estimates of specific antibody concentration from Example 15(c), each dose of the Interval 6 antibody prep contained approximately 400 µg of specific antibody. On day 2 each hamster was predisposed to *C. difficile* infection by the oral administration of 3.0 mg of Clindamycin-HCl (Sigma) in 1 ml of water. On day 3 the hamsters were orally challenged with 1 ml of *C. difficile* inoculum strain ATCC 43596 in sterile saline containing approximately 100 organisms. The animals were then observed for the onset of diarrhea and subsequent death during the treatment period. The results are shown in Table 19.

TABLE 19

Lethality After 12 Days Of Treatment

Treatment Group	Number Animals Alive	Number Animals Dead
Preimmune	0	7
CTAB	6	1
Interval 6	7	0

Treatment of hamsters with orally-administered IgYs against Interval 6 successfully protected 7 out of 7 (100%) of the animals from *C. difficile* disease. One of the hamsters in this group presented with diarrhea which subsequently resolved during the course of treatment. As shown previously in Example 9, antibodies to native toxin A and toxin B were highly protective. In this Example, 6 out of 7 animals survived in the CTAB treatment group. All of the hamsters treated with preimmune sera came down with diarrhea and died. The survivors in both the CTAB and Interval 6 groups remained healthy throughout a 12 day post-treatment period. In particular, 6 out of 7 Interval 6-treated hamsters survived at least 2 weeks after termination of treatment which suggests that these antibodies provide a long-lasting cure. These results represent the first demonstration that antibodies generated against a recombinant region of toxin A can prevent CDAD when administered passively to animals. These results also indicate that antibodies raised against Interval 6 alone may

be sufficient to protect animals from *C. difficile* disease when administered prophylactically.

Previously others had raised antibodies against toxin A by actively immunizing hamsters against a recombinant polypeptide located within the Interval 6 region [Lyerly, D.M., *et al.* (1990) *Curr. Microbiol.* 21:29]. Figure 17 shows schematically the location of the Lyerly, *et al.* intra-Interval 6 recombinant protein (cloned into the pUC vector) in comparison with the complete Interval 6 construct (pMA1870-2680) used herein to generate neutralizing antibodies directed against toxin A. In Figure 17, the solid black oval represents the MBP which is fused to the toxin A Interval 6 in pMA1870-2680.

The Lyerly, *et al.* antibodies (intra-Interval 6) were only able to partially protect hamsters against *C. difficile* infection in terms of survival (4 out of 8 animals survived) and furthermore, these antibodies did not prevent diarrhea in any of the animals. Additionally, animals treated with the intra-Interval 6 antibodies [Lyerly, *et al.* (1990), *supra*] died when treatment was removed.

In contrast, the experiment shown above demonstrates that passive administration of anti-Interval 6 antibodies prevented diarrhea in 6 out of 7 animals and completely prevented death due to CDAD. Furthermore, as discussed above, passive administration of the anti-Interval 6 antibodies provides a long lasting cure (*i.e.*, treatment could be withdrawn without incident).

**b) Therapeutic Treatment Of *C. difficile* Disease: *In Vivo*
Treatment Of An Established *C. difficile* Infection In
Hamsters With Recombinant Interval 6 Antibodies**

The ability of antibodies against recombinant interval 6 of toxin A to therapeutically treat *C. difficile* disease was examined. The experiment was performed essentially as described in Example 10(b). Three groups, each containing seven to eight female Golden Syrian hamsters (100 g each; Charles River) were treated with either preimmune IgY, IgYs against native toxin A and toxin B (CTAB) and IgYs

against Interval 6. The antibodies were prepared as described above as 4X PEG preparations.

The hamsters were first predisposed to *C. difficile* infection with a 3 mg dose of Clindamycin-HCl (Sigma) administered orally in 1 ml of water. Approximately 24 hrs later, the animals were orally challenged with 1 ml of *C. difficile* strain ATCC 43596 in sterile saline containing approximately 200 organisms. One day after infection, the presence of toxin A and B was determined in the feces of the hamsters using a commercial immunoassay kit (Cytoclone A+B EPA, Cambridge Biotech) to verify establishment of infection. Four members of each group were randomly selected and tested. Feces from an uninfected hamster was tested as a negative control. All infected animals tested positive for the presence of toxin according to the manufacturer's procedure. The initiation of treatment then started approximately 24 hr post-infection.

The animals were dosed daily at roughly 4 hr intervals with 1 ml antibody preparation diluted in Ensure® (Ross Labs). The amount of specific antibodies given per dose (determined by affinity purification) was estimated to be about 400 µg of anti-Interval 6 IgY (for animals in the Interval 6 group) and 100 µg and 70 µg of anti-toxin A (Interval 6-specific) and anti-toxin B (Interval 3-specific; see Example 19), respectively, for the CTAB preparation. The animals were treated for 9 days and then observed for an additional 4 days for the presence of diarrhea and death. The results indicating the number of survivors and the number of dead 4 days post-infection are shown in Table 20.

TABLE 20

In vivo Therapeutic Treatment With Interval 6 Antibodies

Treatment Group	Number Animals Alive	Number Animals Dead
Preimmune	4	3
CTAB	8	0
Interval 6	8	0

Antibodies directed against both Interval 6 and CTAB successfully prevented death from *C. difficile* when therapeutically administered 24 hr after infection. This result is significant since many investigators begin therapeutic treatment of hamsters with existing drugs (*e.g.*, vancomycin, phenelfamycins, tiacumicins, etc.) 8 hr post-infection [Swanson, *et al.* (1991) Antimicrobial Agents and Chemotherapy 35:1108 and (1989) J. Antibiotics 42:94].

Forty-two percent of hamsters treated with preimmune IgY died from CDAD. While the anti-Interval 6 antibodies prevented death in the treated hamsters, they did not eliminate all symptoms of CDAD as 3 animals presented with slight diarrhea. In addition, one CTAB-treated and one preimmune-treated animal also had diarrhea 14 days post-infection. These results indicate that anti-Interval 6 antibodies provide an effective means of therapy for CDAD.

EXAMPLE 17

Induction Of Toxin A Neutralizing Antibodies Requires Soluble Interval 6 Protein

As shown in Examples 11(d) and 15, expression of recombinant proteins in *E. coli* may result in the production of either soluble or insoluble protein. If insoluble protein is produced, the recombinant protein is solubilized prior to immunization of animals. To determine whether, one or both of the soluble or insoluble recombinant proteins could be used to generate neutralizing antibodies to toxin A, the following experiment was performed. This example involved a) expression of the toxin A repeats and subfragments of these repeats in *E. coli* using a variety of expression vectors; b) identification of recombinant toxin A repeats and sub-regions to which neutralizing antibodies bind; and c) determination of the neutralization ability of antibodies raised against soluble and insoluble toxin A repeat immunogen.

a) **Expression Of The Toxin A Repeats And
Subfragments Of These Repeats In *E. coli*
Using A Variety Of Expression Vectors**

The Interval 6 immunogen utilized in Examples 15 and 16 was the pMA1870-2680 protein, in which the toxin A repeats are expressed as a soluble fusion protein with the MBP (described in Example 11). Interestingly, expression of this region (from the *SpeI* site to the end of the repeats, see Figure 15B) in three other expression constructs, as either native (pPA1870-2680), poly-His tagged [pPA1870-2680 (H)] or biotin-tagged (pBA1870-2680) proteins resulted in completely insoluble protein upon induction of the bacterial host (see Figure 15B). The host strain BL21 (Novagen) was used for expression of pBA1870-2680 and host strain BL21(DE3) (Novagen) was used for expression of pPA1870-2680 and pPA1870-2680(H). These insoluble proteins accumulated to high levels in inclusion bodies. Expression of recombinant plasmids in *E. coli* host cells grown in 2X YT medium was performed as described [Williams, *et al.* (1995), *supra*].

As summarized in Figure 15B, expression of fragments of the toxin A repeats (as either N-terminal *SpeI*-*EcoRI* fragments, or C-terminal *EcoRI*-end fragments) also yielded high levels of insoluble protein using pGEX (pGA1870-2190), PinPoint™-Xa (pBA1870-2190 and pBA2250-2680) and pET expression systems (pPA1870-2190). The pGEX and pET expression systems are described in Example 11. The PinPoint™-Xa expression system drives the expression of fusion proteins in *E. coli*. Fusion proteins from PinPoint™-Xa vectors contain a biotin tag at the amino-terminal end and can be affinity purified SoftLink™ Soft Release avidin resin (Promega) under mild denaturing conditions (5 mM biotin).

The solubility of expressed proteins from the pPG1870-2190 and pPA1870-2190 expression constructs was determined after induction of recombinant protein expression under conditions reported to enhance protein solubility [These conditions comprise growth of the host at reduced temperature (30°C) and the utilization of high (1 mM IPTG) or low (0.1 mM IPTG) concentrations of inducer [Williams *et al.*

(1995), *supra*]. All expressed recombinant toxin A protein was insoluble under these conditions. Thus, expression of these fragments of the toxin A repeats in pET and pGEX expression vectors results in the production of insoluble recombinant protein even when the host cells are grown at reduced temperature and using lower concentrations of the inducer. Although expression of these fragments in pMal vectors yielded affinity purifiable soluble fusion protein, the protein was either predominantly insoluble (pMA1870-2190) or unstable (pMA2250-2650). Attempts to solubilize expressed protein from the pMA1870-2190 expression construct using reduced temperature or lower inducer concentration (as described above) did not improve fusion protein solubility.

Collectively, these results demonstrate that expression of the toxin A repeat region in *E. coli* results in the production of insoluble recombinant protein, when expressed as either large (aa 1870-2680) or small (aa 1870-2190 or aa 2250-2680) fragments, in a variety of expression vectors (native or poly-his tagged pET, pGEX or PinPoint™- Xa vectors), utilizing growth conditions shown to enhance protein solubility. The exception to this rule were fusions with the MBP, which enhanced protein solubility, either partially (pMA1870-2190) or fully (pMA1870-2680).

b) Identification Of Recombinant Toxin A Repeats And Sub-Regions To Which Neutralizing Antibodies Bind

Toxin A repeat regions to which neutralizing antibodies bind were identified by utilizing recombinant toxin A repeat region proteins expressed as soluble or insoluble proteins to deplete protective antibodies from a polyclonal pool of antibodies against native *C. difficile* toxin A. An *in vivo* assay was developed to evaluate proteins for the ability to bind neutralizing antibodies.

The rationale for this assay is as follows. Recombinant proteins were first pre-mixed with antibodies against native toxin A (CTA antibody; generated in Example 8) and allowed to react. Subsequently, *C. difficile* toxin A was added at a concentration lethal to hamsters and the mixture was administered to hamsters via IP injection. If

the recombinant protein contains neutralizing epitopes, the CTA antibodies would lose their ability to bind toxin A resulting in diarrhea and/or death of the hamsters.

The assay was performed as follows. The lethal dose of toxin A when delivered orally to nine 40 to 50 g Golden Syrian hamsters (Sasco) was determined to be 10 to 30 μ g. The PEG-purified CTA antibody preparation was diluted to 0.5X concentration (*i.e.*, the antibodies were diluted at twice the original yolk volume) in 0.1 M carbonate buffer, pH 9.5. The antibodies were diluted in carbonate buffer to protect them from acid degradation in the stomach. The concentration of 0.5X was used because it was found to be the lowest effective concentration against toxin A. The concentration of Interval 6-specific antibodies in the 0.5X CTA prep was estimated to be 10-15 μ g/ml (estimated using the method described in Example 15).

The inclusion body preparation [insoluble Interval 6 protein; pPA1870-2680(H)] and the soluble Interval 6 protein [pMA1870-2680; see Figure 15] were both compared for their ability to bind to neutralizing antibodies against *C. difficile* toxin A (CTA). Specifically, 1 to 2 mg of recombinant protein was mixed with 5 ml of a 0.5X CTA antibody prep (estimated to contain 60-70 μ g of Interval 6-specific antibody). After incubation for 1 hr at 37°C, CTA (Tech Lab) at a final concentration of 30 μ g/ml was added and incubated for another 1 hr at 37°C. One ml of this mixture containing 30 μ g of toxin A (and 10-15 μ g of Interval 6-specific antibody) was administered orally to 40-50 g Golden Syrian hamsters (Sasco). Recombinant proteins that result in the loss of neutralizing capacity of the CTA antibody would indicate that those proteins contain neutralizing epitopes. Preimmune and CTA antibodies (both at 0.5X) without the addition of any recombinant protein served as negative and positive controls, respectively.

Two other inclusion body preparations, both expressed as insoluble products in the pET vector, were tested; one containing a different insert (toxin B fragment) other than Interval 6 called pPB1850-2070 (see Figure 18) which serves as a control for insoluble Interval 6, the other was a truncated version of the Interval 6 region called pPA1870-2190 (see Figure 15B). The results of this experiment are shown in Table 21.

TABLE 21

Binding Of Neutralizing Antibodies By Soluble Interval 6 Protein Study Outcome After 24 Hours

Treatment Group ¹	Healthy ²	Diarrhea ³	Dead ⁴
Preimmune Ab	0	3	2
CTA Ab	4	1	0
CTA Ab + Int 6 (soluble)	1	2	2
CTA Ab + Int 6 (insoluble)	5	0	0
CTA Ab + pPB1850-2070	5	0	0
CTA Ab + pPA1870-2190	5	0	0

¹ *C. difficile* toxin A (CTA) was added to each group.² Animals showed no signs of illness.³ Animals developed diarrhea but did not die.⁴ Animals developed diarrhea and died.

Preimmune antibody was ineffective against toxin A, while anti-CTA antibodies at a dilute 0.5X concentration almost completely protected the hamsters against the enterotoxic effects of CTA. The addition of recombinant proteins pPB1850-2070 or pPA1870-2190 to the anti-CTA antibody had no effect upon its protective ability, indicating that these recombinant proteins do not bind to neutralizing antibodies. On the other hand, recombinant Interval 6 protein was able to bind to neutralizing anti-CTA antibodies and neutralized the *in vivo* protective effect of the anti-CTA antibodies. Four out of five animals in the group treated with anti-CTA antibodies mixed with soluble Interval 6 protein exhibited toxin associated toxicity (diarrhea and death). Moreover, the results showed that Interval 6 protein must be expressed as a soluble product in order for it to bind to neutralizing anti-CTA antibodies since the addition of insoluble Interval 6 protein had no effect on the neutralizing capacity of the CTA antibody prep.

c) **Determination Of Neutralization Ability Of Antibodies
Raised Against Soluble And Insoluble Toxin A Repeat
Immunogen**

To determine if neutralizing antibodies are induced against solubilized inclusion bodies, insoluble toxin A repeat protein was solubilized and specific antibodies were raised in chickens. Insoluble pPA1870-2680 protein was solubilized using the method described in Williams *et al.* (1995), *supra*. Briefly, induced cultures (500 ml) were pelleted by centrifugation at 3,000 X g for 10 min at 4°C. The cell pellets were resuspended thoroughly in 10 ml of inclusion body sonication buffer (25 mM HEPES pH 7.7, 100 mM KCl, 12.5 mM MgCl₂, 20% glycerol, 0.1% (v/v) Nonidet P-40, 1 mM DTT). The suspension was transferred to a 30 ml non-glass centrifuge tube. Five hundred µl of 10 mg/ml lysozyme was added and the tubes were incubated on ice for 30 min. The suspension was then frozen at -70°C for at least 1 hr. The suspension was thawed rapidly in a water bath at room temperature and then placed on ice. The suspension was then sonicated using at least eight 15 sec bursts of the microprobe (Branson Sonicator Model No. 450) with intermittent cooling on ice.

The sonicated suspension was transferred to a 35 ml Oakridge tube and centrifuged at 6,000 X g for 10 min at 4°C to pellet the inclusion bodies. The pellet was washed 2 times by pipetting or vortexing in fresh, ice-cold RIPA buffer [0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate in TBS (25 mM Tris-Cl pH 7.5, 150 mM NaCl)]. The inclusion bodies were recentrifuged after each wash. The inclusion bodies were dried and transferred using a small metal spatula to a 15 ml tube (Falcon). One ml of 10% SDS was added and the pellet was solubilized by gently pipetting the solution up and down using a 1 ml micropipettor. The solubilization was facilitated by heating the sample to 95°C when necessary.

Once the inclusion bodies were in solution, the samples were diluted with 9 volumes of PBS. The protein solutions were dialyzed overnight against a 100-fold volume of PBS containing 0.05% SDS at room temperature. The dialysis buffer was then changed to PBS containing 0.01% SDS and the samples were dialyzed for several hours to overnight at room temperature. The samples were stored at 4°C until used. Prior to further use, the samples were warmed to room temperature to allow any precipitated SDS to go back into solution.

The inclusion body preparation was used to immunize hens. The protein was dialyzed into PBS and emulsified with approximately equal volumes of CFA for the initial immunization or IFA for subsequent booster immunizations. On day zero, for each of the recombinant recombinant preparations, two egg laying white Leghorn hens were each injected at multiple sites (IM and SC) with 1 ml of recombinant protein-adjuvant mixture containing approximately 0.5-1.5 mg of recombinant protein. Booster immunizations of 1.0 mg were given of days 14 and day 28. Eggs were collected on day 32 and the antibody isolated using PEG as described in Example 14(a). High titers of toxin A specific antibodies were present (as assayed by ELISA, using the method described in Example 13). Titers were determined for both antibodies against recombinant polypeptides pPA1870-2680 and pMA1870-2680 and were found to be comparable at > 1:62,500.

Antibodies against soluble Interval 6 (pMA1870-2680) and insoluble Interval 6 [(inclusion body), pPA1870-2680] were tested for neutralizing ability against toxin A using the *in vivo* assay described in Example 15(b). Preimmune antibodies and antibodies against toxin A (CTA) served as negative and positive controls, respectively. The results are shown in Table 22.

TABLE 22

Neutralization Of Toxin A By Antibodies Against
Soluble Interval 6 Protein Study Outcome After 24 Hours

Antibody Treatment Group	Healthy ¹	Diarrhea ²	Dead ³
Preimmune	1	0	4
CTA	5	0	0
Interval 6 (Soluble) ⁴	5	0	0
Interval 6 (Insoluble)	0	2	3

¹ Animals showed no sign of illness.

² Animal developed diarrhea but did not die.

³ Animal developed diarrhea and died.

⁴ 400 µg/ml.

Antibodies raised against native toxin A were protective while preimmune antibodies had little effect. As found using the *in vitro* CHO assay [described in

Example 8(d)] where antibodies raised against the soluble Interval 6 could partially neutralize the effects of toxin A, here they were able to completely neutralize toxin A *in vivo*. In contrast, the antibodies raised against the insoluble Interval 6 was unable to neutralize the effects of toxin A *in vivo* as shown above (Table 22) and *in vitro* as shown in the CHO assay [described in Example 8(d)].

These results demonstrate that soluble toxin A repeat immunogen is necessary to induce the production of neutralizing antibodies in chickens, and that the generation of such soluble immunogen is obtained only with a specific expression vector (pMal) containing the toxin A region spanning aa 1870-2680. That is to say, insoluble protein that is subsequently solubilized does not result in a toxin A antigen that will elicit a neutralizing antibody.

EXAMPLE 18

Cloning And Expression Of The *C. difficile* Toxin B Gene

The toxin B gene has been cloned and sequenced; the amino acid sequence deduced from the cloned nucleotide sequence predicts a MW of 269.7 kD for toxin B [Barroso *et al.*, Nucl. Acids Res. 18:4004 (1990)]. The nucleotide sequence of the coding region of the entire toxin B gene is listed in SEQ ID NO:9. The amino acid sequence of the entire toxin B protein is listed in SEQ ID NO:10. The amino acid sequence consisting of amino acid residues 1850 through 2360 of toxin B is listed in SEQ ID NO:11. The amino acid sequence consisting of amino acid residues 1750 through 2360 of toxin B is listed in SEQ ID NO:12.

Given the expense and difficulty of isolating native toxin B protein, it would be advantageous to use simple and inexpensive procaryotic expression systems to produce and purify high levels of recombinant toxin B protein for immunization purposes. Ideally, the isolated recombinant protein would be soluble in order to preserve native antigenicity, since solubilized inclusion body proteins often do not fold into native conformations. Indeed as shown in Example 17, neutralizing antibodies against recombinant toxin A were only obtained when soluble recombinant toxin A

polypeptides were used as the immunogen. To allow ease of purification, the recombinant protein should be expressed to levels greater than 1 mg/liter of *E. coli* culture.

To determine whether high levels of recombinant toxin B protein could be produced in *E. coli*, fragments of the toxin B gene were cloned into various prokaryotic expression vectors, and assessed for the ability to express recombinant toxin B protein in *E. coli*. This Example involved (a) cloning of the toxin B gene and (b) expression of the toxin B gene in *E. coli*.

a) Cloning Of The Toxin B Gene

The toxin B gene was cloned using PCR amplification from *C. difficile* genomic DNA. Initially, the gene was cloned in two overlapping fragments, using primer pairs P5/P6 and P7/P8. The location of these primers along the toxin B gene is shown schematically in Figure 18. The sequence of each of these primers is: P5: 5' TAGAAAAAATGGCAAATGT 3' (SEQ ID NO:11); P6: 5' TTTCATCTTGTA GAGTCAAAG 3' (SEQ ID NO:12); P7: 5' GATGCCACAAGATGATTAGTG 3' (SEQ ID NO:13); and P8: 5' CTAATTGAGCTGTATCAGGATC 3' (SEQ ID NO:14).

Figure 18 also shows the location of the following primers along the toxin B gene: P9 which consists of the sequence 5' CGGAATTCCTAGAAAAAATGGCAA ATG 3' (SEQ ID NO:15); P10 which consists of the sequence 5' GCTCTAGAATGA CCATAAGCTAGCCA 3' (SEQ ID NO:16); P11 which consists of the sequence 5' CGGAATTCGAGTTGGTAGAAAGGTGGA 3' (SEQ ID NO:17); P13 which consists of the sequence 5' CGGAATTCGGTTATTATCTTAAGGATG 3' (SEQ ID NO:18); and P14 which consists of the sequence 5' CGGAATTCTTGATAACTGGAT TTGTGAC 3' (SEQ ID NO:19). The amino acid sequence consisting of amino acid residues 1852 through 2362 of toxin B is listed in SEQ ID NO:20. The amino acid sequence consisting of amino acid residues 1755 through 2362 of toxin B is listed in SEQ ID NO:21.

Clostridium difficile VPI strain 10463 was obtained from the American Type Culture Collection (ATCC 43255) and grown under anaerobic conditions in brain-heart infusion medium (Becton Dickinson). High molecular-weight *C. difficile* DNA was isolated essentially as described [Wren and Tabaqchali (1987) J. Clin. Microbiol., 25:2402], except 1) 100 µg/ml proteinase K in 0.5% SDS was used to disrupt the bacteria and 2) cetyltrimethylammonium bromide (CTAB) precipitation [as described by Ausubel *et al.*, Eds., *Current Protocols in Molecular Biology*, Vol. 2 (1989) Current Protocols] was used to remove carbohydrates from the cleared lysate. Briefly, after disruption of the bacteria with proteinase K and SDS, the solution is adjusted to approximately 0.7 M NaCl by the addition of a 1/7 volume of 5M NaCl. A 1/10 volume of CTAB/NaCl (10% CTAB in 0.7 M NaCl) solution was added and the solution was mixed thoroughly and incubated 10 min at 65°C. An equal volume of chloroform/isoamyl alcohol (24:1) was added and the phases were thoroughly mixed. The organic and aqueous phases were separated by centrifugation in a microfuge for 5 min. The aqueous supernatant was removed and extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The phases were separated by centrifugation in a microfuge for 5 min. The supernatant was transferred to a fresh tube and the DNA was precipitated with isopropanol. The DNA precipitate was pelleted by brief centrifugation in a microfuge. The DNA pellet was washed with 70% ethanol to remove residual CTAB. The DNA pellet was then dried and redissolved in TE buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA). The integrity and yield of genomic DNA was assessed by comparison with a serial dilution of uncut lambda DNA after electrophoresis on an agarose gel.

Toxin B fragments were cloned by PCR utilizing a proofreading thermostable DNA polymerase [native *Pfu* polymerase (Stratagene)]. The high fidelity of this polymerase reduces the mutation problems associated with amplification by error prone polymerases (*e.g.*, *Taq* polymerase). PCR amplification was performed using the PCR primer pairs P5 (SEQ ID NO:11) with P6 (SEQ ID NO:12) and P7 (SEQ ID NO:13) with P8 (SEQ ID NO:14) in 50 µl reactions containing 10 mM Tris-HCl pH8.3, 50

mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP, 0.2 μM each primer, and 50 ng *C. difficile* genomic DNA. Reactions were overlaid with 100 μl mineral oil, heated to 94°C for 4 min, 0.5 μl native *Pfu* polymerase (Stratagene) was added, and the reactions were cycled 30 times at 94°C for 1 min, 50°C for 1 min, 72°C (2 min for each kb of sequence to be amplified), followed by 10 min at 72°C. Duplicate reactions were pooled, chloroform extracted, and ethanol precipitated. After washing in 70% ethanol, the pellets were resuspended in 50 μl TE buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA).

The P5/P6 amplification product was cloned into pUC19 as outlined below. 10 μl aliquots of DNA were gel purified using the Prep-a-Gene kit (BioRad), and ligated to *Sma*I restricted pUC19 vector. Recombinant clones were isolated and confirmed by restriction digestion using standard recombinant molecular biology techniques (Sambrook *et al.*, 1989). Inserts from two independent isolates were identified in which the toxin B insert was oriented such that the vector *Bam*HI and *Sac*I sites were 5' and 3' oriented, respectively (pUCB10-1530). The insert-containing *Bam*HI/*Sac*I fragment was cloned into similarly cut pET23a-c vector DNA, and protein expression was induced in small scale cultures (5 ml) of identified clones.

Total protein extracts were isolated, resolved on SDS-PAGE gels, and toxin B protein identified by Western analysis utilizing a goat anti-toxin B affinity purified antibody (Tech Lab). Procedures for protein induction, SDS-PAGE, and Western blot analysis were performed as described in Williams *et al.* (1995), *supra*. In brief, 5 ml cultures of bacteria grown in 2XYT containing 100 μg/ml ampicillin containing the appropriate recombinant clone were induced to express recombinant protein by addition of IPTG to 1mM. The *E. coli* hosts used were: BL21(DE3) or BL21(DE3)LysS (Novagen) for pET plasmids.

Cultures were induced by the addition of IPTG to a final concentration of 1.0 mM when the cell density reached 0.5 OD₆₀₀, and induced protein was allowed to accumulate for two hrs after induction. Protein samples were prepared by pelleting 1 ml aliquots of bacteria by centrifugation (1 min in microfuge), and resuspension of the

pelleted bacteria in 150 µl of 2X SDS-PAGE sample buffer (0.125 mM Tris-HCl pH 6.8, 2 mM EDTA, 6% SDS, 20% glycerol, 0.025% bromophenol blue; β-mercaptoethanol is added to 5% before use). The samples were heated to 95°C for 5 min, then cooled and 5 or 10 µls loaded on 7.5% SDS-PAGE gels. High molecular weight protein markers (BioRad) were also loaded, to allow estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining the gels with Coomassie Blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein. The MW of induced toxin B reactive protein allowed the integrity of the toxin B reading frame to be determined.

The pET23b recombinant (pPB10-1530) expressed high MW recombinant toxin B reactive protein, consistent with predicted values. This confirmed that frame terminating errors had not occurred during PCR amplification. A pET23b expression clone containing the 10-1750aa interval of the toxin B gene was constructed, by fusion of the *EcoRV-SpeI* fragment of the P7/P8 amplification product to the P5-*EcoRV* interval of the P5/P6 amplification product (see Figure 18) in pPB10-1530. The integrity of this clone (pPB10-1750) was confirmed by restriction mapping, and Western blot detection of expressed recombinant toxin B protein. Levels of induced protein from both pPB10-1530 and pPB10-1750 were too low to facilitate purification of usable amounts of recombinant toxin B protein. The remaining 1750-2360 aa interval was directly cloned into pMAL or pET expression vectors from the P7/P8 amplification product as described below.

b) Expression Of The Toxin B Gene

i) Overview Of Expression Methodologies

Fragments of the toxin B gene were expressed as either native or fusion proteins in *E. coli*. Native proteins were expressed in either the pET23a-c or pET16b expression vectors (Novagen). The pET23 vectors contain an extensive polylinker sequence in all three reading frames (a-c vectors) followed by a C-terminal poly-histidine repeat. The pET16b vector contains a N-terminal poly-histidine sequence

immediately 5' to a small polylinker. The poly-histidine sequence binds to Ni-Chelate columns and allows affinity purification of tagged target proteins [Williams *et al.* (1995), *supra*]. These affinity tags are small (10 aa for pET16b, 6 aa for pET23) allowing the expression and affinity purification of native proteins with only limited amounts of foreign sequences.

An N-terminal histidine-tagged derivative of pET16b containing an extensive cloning cassette was constructed to facilitate cloning of N-terminal poly-histidine tagged toxin B expressing constructs. This was accomplished by replacement of the promoter region of the pET23a and b vectors with that of the pET16b expression vector. Each vector was restricted with *Bgl*II and *Nde*I, and the reactions resolved on a 1.2 % agarose gel. The pET16b promoter region (contained in a 200 bp *Bgl*II-*Nde*I fragment) and the promoter-less pET23 a or b vectors were cut from the gel, mixed and Prep-A-Gene (BioRad) purified. The eluted DNA was ligated, and transformants screened for promoter replacement by *Nco*I digestion of purified plasmid DNA (the pET16b promoter contains this site, the pET23 promoter does not). These clones (denoted pETHisa or b) contain the pET16b promoter (consisting of a pT7-lac promoter, ribosome binding site and poly-histidine (10aa) sequence) fused at the *Nde*I site to the extensive pET23 polylinker.

All MBP fusion proteins were constructed and expressed in the pMALTM-c or pMALTM-p2 vectors (New England Biolabs) in which the protein of interest is expressed as a C-terminal fusion with MBP. All pET plasmids were expressed in either the BL21(DE3) or BL21(DE3)LysS expression hosts, while pMal plasmids were expressed in the BL21 host.

Large scale (500 mls to 1 liter) cultures of each recombinant were grown in 2X YT broth, induced, and soluble protein fractions were isolated as described [Williams, *et al.* (1995), *supra*]. The soluble protein extracts were affinity chromatographed to isolate recombinant fusion protein, as described [Williams *et al.*, (1995) *supra*]. In brief, extracts containing tagged pET fusions were chromatographed on a nickel chelate column, and eluted using imidazole salts or low pH (pH 4.0) as described by

obtained from pMAL expression constructs spanning the entire toxin B gene, and yields of full length soluble fusion protein ranged from an estimated 1 mg/liter culture (pMB1100-1530) to greater than 20 mg/liter culture (pMB1750-2360).

Representative purifications of MBP and poly-histidine-tagged toxin B recombinants are shown in Figures 21 and 22. Figure 21 shows a Coomassie Blue stained 7.5% SDS-PAGE gel on which various protein samples extracted from bacteria harboring pMB1850-2360 were electrophoresed. Samples were loaded as follows: Lane 1: protein extracted from uninduced culture; Lane 2: induced culture protein; Lane 3: total protein from induced culture after sonication; Lane 4: soluble protein; and Lane 5: eluted affinity purified protein. Figure 22 depicts the purification of recombinant proteins expressed in bacteria harboring either pPB1850-2360 (Lanes 1-3) or pPB1750-2360 (Lanes 4-6). Samples were loaded as follows: uninduced total protein (Lanes 1 and 4); induced total protein (Lanes 2 and 5); and eluted affinity purified protein (Lanes 3 and 6). The broad range molecular weight protein markers (BioRad) are shown in Lane 7.

Thus, although high level expression was not attained using large expression constructs from the toxin B gene, usable levels of recombinant protein were obtained by isolating induced protein from a series of smaller pMAL expression constructs that span the entire toxin B gene.

These results represent the first demonstration of the feasibility of expressing recombinant toxin B protein to high levels in *E. coli*. As well, expression of small regions of the putative ligand binding domain (repeat region) of toxin B as native protein yielded insoluble protein, while large constructs, or fusions to MBP were soluble (Figure 19), demonstrating that specific methodologies are necessary to produce soluble fusion protein from this interval.

iii) Clone Construction And Expression

Details

A portion of the toxin B gene containing the toxin B repeat region [amino acid residues 1852-2362 of toxin B (SEQ ID NO:20)] was isolated by PCR amplification of this interval of the toxin B gene from *C. difficile* genomic DNA. The sequence, and location within the toxin B gene, of the two PCR primers [P7 (SEQ ID NO:13) and P8 (SEQ ID NO:14)] used to amplify this region are shown in Figure 18.

DNA from the PCR amplification was purified by chloroform extraction and ethanol precipitation as described above. The DNA was restricted with *SpeI*, and the cleaved DNA was resolved by agarose gel electrophoresis. The restriction digestion with *SpeI* cleaved the 3.6 kb amplification product into a 1.8 kb doublet band. This doublet band was cut from the gel and mixed with appropriately cut, gel purified pMALc or pET23b vector. These vectors were prepared by digestion with *HindIII*, filling in the overhanging ends using the Klenow enzyme, and cleaving with *XbaI* (pMALc) or *NheI* (pET23b). The gel purified DNA fragments were purified using the Prep-A-Gene kit (BioRad) and the DNA was ligated, transformed and putative recombinant clones analyzed by restriction mapping.

pET and pMal clones containing the toxin B repeat insert (aa interval 1750-2360 of toxin B) were verified by restriction mapping, using enzymes that cleaved specific sites within the toxin B region. In both cases fusion of the toxin B *SpeI* site with either the compatible *XbaI* site (pMal) or compatible *NheI* site (pET) is predicted to create an in frame fusion. This was confirmed in the case of the pMB1750-2360 clone by DNA sequencing of the clone junction and 5' end of the toxin B insert using a MBP specific primer (New England Biolabs). In the case of the pET construct, the fusion of the blunt ended toxin B 3' end to the filled *HindIII* site should create an in-frame fusion with the C-terminal poly-histidine sequence in this vector. The pPB1750-2360 clone selected had lost, as predicted, the *HindIII* site at this clone junction; this eliminated the possibility that an additional adenosine residue was added to the 3' end of the PCR product by a terminal transferase activity of the *Pfu* polymerase, since

fusion of this adenosine residue to the filled *Hind*III site would regenerate the restriction site (and was observed in several clones).

One liter cultures of each expression construct were grown, and fusion protein purified by affinity chromatography on either an amylose resin column (pMAL
5 constructs; resin supplied by New England Biolabs) or Ni-chelate column (pET constructs; resin supplied by Qiagen or Novagen) as described [Williams *et al.* (1995), *supra*]. The integrity and purity of the fusion proteins were determined by Coomassie staining of SDS-PAGE protein gels as well as Western blot analysis with either an
10 affinity purified goat polyclonal antiserum (Tech Lab), or a chicken polyclonal PEG prep, raised against the toxin B protein (CTB) as described above in Example 8. In both cases, affinity purification resulted in yields in excess of 20 mg protein per liter culture, of which greater than 90% was estimated to be full-length recombinant protein. It should be noted that the poly-histidine affinity tagged protein was released from the Qiagen Ni-NTA resin at low imidazole concentration (60 mM), necessitating
15 the use of a 40 mM imidazole rather than a 60 mM imidazole wash step during purification.

A periplasmically secreted version of pMB1750-2360 was constructed by replacement of the promoter and MBP coding region of this construct with that from a related vector (pMALTM-p2; New England Biolabs) in which a signal sequence is
20 present at the N-terminus of the MBP, such that fusion protein is exported. This was accomplished by substituting a *Bgl*III-*Eco*RV promoter fragment from pMAL-p2 into pMB1750-2360. The yields of secreted, affinity purified protein (recovered from osmotic shock extracts as described by Riggs in *Current Protocols in Molecular Biology*, Vol. 2, Ausubel, *et al.*, Eds. (1989), Current Protocols, pp. 16.6.1 - 16.6.14]
25 from this vector (pMBp1750-2360) were 6.5 mg/liter culture, of which 50% was estimated to be full-length fusion protein.

The interval was also expressed in two non-overlapping fragments. pMB1750-1970 was constructed by introduction of a frameshift into pMB1750-2360, by restriction with *Hind*III, filling in the overhanging ends and religation of the plasmid.

Recombinant clones were selected by loss of the *HindIII* site, and further restriction map analysis. Recombinant protein expression from this vector was more than 20 mg/liter of greater than 90% pure protein.

5 The complementary region was expressed in pMB1970-2360. This construct was created by removal of the 1750-1970 interval of pMB1750-2360. This was accomplished by restriction of this plasmid with *EcoRI* (in the pMalc polylinker 5' to the insert) and *III*, filling in the overhanging ends, and religation of the plasmid. The resultant plasmid, pMB1970-2360, was made using both intracellularly and secreted versions of the pMB1750-2360 vector.

10 No fusion protein was secreted in the pMBp1970-2360 version, perhaps due to a conformational constraint that prevents export of the fusion protein. However, the intracellularly expressed vector produced greater than 40mg/liter of greater than 90% full-length fusion protein.

15 Constructs to precisely express the toxin B repeats in either pMalc (pMB1850-2360) or pET16b (pPB1850-2360) were constructed as follows. The DNA interval including the toxin B repeats was PCR amplified as described above utilizing PCR primers P14 (SEQ ID NO:19) and P8 (SEQ ID NO:14). Primer P14 adds a *EcoRI* site immediately flanking the start of the toxin B repeats.

20 The amplified fragment was cloned into the pT7 Blue T-vector (Novagen) and recombinant clones in which single copies of the PCR fragment were inserted in either orientation were selected (pT71850-2360) and confirmed by restriction mapping. The insert was excised from two appropriately oriented independently isolated pT71850-2360 plasmids, with *EcoRI* (5' end of repeats) and *PstI* (in the flanking polylinker of the vector), and cloned into *EcoRI/PstI* cleaved pMalc vector. The resulting construct
25 (pMB1850-2360) was confirmed by restriction analysis, and yielded 20 mg/l of soluble fusion protein [greater than 90% intact (*i.e.*, nondegraded)] after affinity chromatography. Restriction of this plasmid with *HindIII* and religation of the vector resulted in the removal of the 1970-2360 interval. The resultant construct (pMB1850-1970) expressed greater than 70 mg/liter of 90% full length fusion protein.

The pPB1850-2360 construct was made by cloning a *EcoRI* (filled with Klenow)-*BamHI* fragment from a pT71850-2360 vector (opposite orientation to that used in the pMB1850-2360 construction) into *NdeI* (filled)/*BamHI* cleaved pET16b vector. Yields of affinity purified soluble fusion protein were 15 mg/liter, of greater than 90% full length fusion protein.

Several smaller expression constructs from the 1750-2070 interval were also constructed in His-tagged pET vectors, but expression of these plasmids in the BL21 (DE3) host resulted in the production of high levels of mostly insoluble protein (see Table 23 and Figure 19). These constructs were made as follows.

pPB1850-1970 was constructed by cloning a *BglII-HindIII* fragment of pPB1850-2360 into *BglII/HindIII* cleaved pET23b vector. pPB1850-2070 was constructed by cloning a *BglII-PvuII* fragment of pPB1850-2360 into *BglII/HincII* cleaved pET23b vector. pPB1750-1970(c) was constructed by removal of the internal *HindIII* fragment of a pPB1750-2360 vector in which the vector *HindIII* site was regenerated during cloning (presumably by the addition of an A residue to the amplified PCR product by terminal transferase activity of *Pfu* polymerase). The pPB1750-1970(n) construct was made by insertion of the insert containing the *NdeI-HindIII* fragment of pPB1750-2360 into identically cleaved pETHisb vector. All constructs were confirmed by restriction digestion.

An expression construct that directs expression of the 10-470 aa interval of toxin B was constructed in the pMalc vector as follows. A *NheI* (a site 5' to the insert in the pET23 vector)-*AflIII* (filled) fragment of the toxin B gene from pPB10-1530 was cloned into *XbaI* (compatible with *NheI*)/*HindIII* (filled) pMalc vector. The integrity of the construct (pMB10-470) was verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer (New England Biolabs). However, all expressed protein was degraded to the MBP monomer MW.

A second construct spanning this interval (aa 10-470) was constructed by cloning the PCR amplification product from a reaction containing the P9 (SEQ ID NO:15) and P10 (SEQ ID NO:16) primers (Figure 18) into the pETHisa vector. This

was accomplished by cloning the PCR product as an *EcoRI*-blunt fragment into *EcoRI*-*HincII* restricted vector DNA; recombinant clones were verified by restriction mapping. Although this construct (pPB10-520) allowed expression and purification (utilizing the N-terminal polyhistidine affinity tag) of intact fusion protein, yields were estimated at less than 500 µg per liter culture.

Higher yield of recombinant protein from this interval (aa 10-520) were obtained by expression of the interval in two overlapping clones. The 10-330aa interval was cloned in both pETHisa and pMalc vectors, using the *BamHI*-*AflIII* (filled) DNA fragment from pPB10-520. This fragment was cloned into *BamHI*-*HindIII* (filled) restricted pMalc or *BamHI*-*HincII* restricted pETHisa vector. Recombinant clones were verified by restriction mapping. High level expression of either insoluble (pET) or soluble (pMal) fusion protein was obtained. Total yields of fusion protein from the pMB10-330 construct (Figure 18) were 20 mg/liter culture, of which 10% was estimated to be full-length fusion protein. Although yields of this interval were higher and >90% full-length recombinant protein produced when expressed from the pET construct, the pMal fusion was utilized since the expressed protein was soluble and thus more likely to have the native conformation.

The pMB260-520 clone was constructed by cloning *EcoRI*-*XbaI* cleaved amplified DNA from a PCR reaction containing the P11 (SEQ ID NO:17) and P10 (SEQ ID NO:16) DNA primers (Figure 18) into similarly restricted pMalc vector. Yields of affinity purified protein were 10 mg/liter, of which approximately 50% was estimated to be full-length recombinant protein.

The aa510-1110 interval was expressed as described below. This entire interval was expressed as a pMal fusion by cloning the *NheI*-*HindIII* fragment of pUCB10-1530 into *XbaI*-*HindIII* cleaved pMalc vector. The integrity of the construct (pMB510-1110) was verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer. The yield of affinity purified protein was 25 mg/liter culture, of which 5% was estimated to be full-length fusion protein (1 mg/liter).

To attempt to obtain higher yields, this region was expressed in two fragments (aa510-820, and 820-1110) in the pMalc vector. The pMB510-820 clone was constructed by insertion of a *SacI* (in the pMalc polylinker 5' to the insert)-*HpaI* DNA fragment from pMB510-1110 into *SacI/StuI* restricted pMalc vector. The pMB820-1110 vector was constructed by insertion of the *HpaI-HindIII* fragment of pUCB10-1530 into *BamHI* (filled)/*HindIII* cleaved pMalc vector. The integrity of these constructs were verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer.

Recombinant protein expressed from the pMB510-820 vector was highly unstable. However, high levels (20 mg/liter) of >90% full-length fusion protein were obtained from the pMB820-1105 construct. The combination of partially degraded pMB510-1110 protein (enriched for the 510-820 interval) with the pMB820-1110 protein provides usable amounts of recombinant antigen from this interval.

The aa1100-1750 interval was expressed as described below. The entire interval was expressed in the pMalc vector from a construct in which the *AccI*(filled)-*SpeI* fragment of pPB10-1750 was inserted into *StuI/XbaI* (*XbaI* is compatible with *SpeI*; *StuI* and filled *AccI* sites are both blunt ended) restricted pMalc. The integrity of this construct (pMB1100-1750) was verified by restriction mapping and DNA sequencing of the clone junction using a MBP specific DNA primer. Although 15 mg/liter of affinity purified protein was isolated from cells harboring this construct, the protein was greater than 99% degraded to MBP monomer size.

A smaller derivative of pMB1100-1750 was constructed by restriction of pMB1100-1750 with *AflII* and *SalI* (in the pMalc polylinker 3' to the insert), filling in the overhanging ends, and religating the plasmid. The resultant clone (verified by restriction digestion and DNA sequencing) has deleted the aa1530-1750 interval, was designated pMB1100-1530. pMB1100-1530 expressed recombinant protein at a yield of greater than 40 mg/liter, of which 5% was estimated to be full-length fusion protein.

Three constructs were made to express the remaining interval. Initially, a *Bsp*HI (filled)-*Spe*I fragment from pPB10-1750 was cloned into *Eco*RI(filled)/*Xba*I cleaved pMalc vector. The integrity of this construct (pMB1570-1750) was verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer. Expression of recombinant protein from this plasmid was very low, approximately 3 mg affinity purified protein per liter, and most was degraded to MBP monomer size. This region was subsequently expressed from a PCR amplified DNA fragment. A PCR reaction utilizing primers P13 [SEQ ID NO:18; P13 was engineered to introduce an *Eco*RI site 5' to amplified toxin B sequences] and P8 (SEQ ID NO:14) was performed on *C. difficile* genomic DNA as described above. The amplified fragment was cleaved with *Eco*RI and *Spe*I, and cloned into *Eco*RI/*Xba*I cleaved pMalc vector. The resultant clone (pMB1530-1750) was verified by restriction map analysis, and recombinant protein was expressed and purified. The yield was greater than 20 mg protein per liter culture and it was estimated that 25% was full-length fusion protein; this was a significantly higher yield than the original pMB1570-1750 clone. The insert of pMB1530-1750 (in a *Eco*RI-*Sal*I fragment) was transferred to the pETHisa vector (*Eco*RI/*Xho*I cleaved, *Xho*I and *Sal*I ends are compatible). No detectable fusion protein was purified on Ni-Chelate columns from soluble lysates of cells induced to express fusion protein from this construct.

TABLE 23

Summary Of Toxin B Expression Constructs*

Clone	Affinity Tag	Yield (mg/liter)	% Full Length
pPB10-1750	none	low (estimated from Western blot hyb.)	?
pPB10-1530	none	low (as above)	?
pMB10-470	MBP	15mg/l	0%
pPB10-520	poly-his	0.5mg/l	20%
pPB10-330	poly-his	>20mg/l (insoluble)	90%
pMB10-330	MBP	20mg/l	10%
pMB260-520	MBP	10mg/l	50%

TABLE 23

Summary Of Toxin B Expression Constructs*

Clone	Affinity Tag	Yield (mg/liter)	% Full Length
<i>pMB510-1110</i>	<i>MBP</i>	<i>25mg/l</i>	<i>5%</i>
pMB510-820	MBP	degraded (by Western blot hyb)	
<i>pMB820-1110</i>	<i>MBP</i>	<i>20mg/l</i>	<i>90%</i>
pMB1100-1750	MBP	15mg/l	0%
<i>pMB1100-1530</i>	<i>MBP</i>	<i>40mg/l</i>	<i>5%</i>
pMB1570-1750	MBP	3mg/l	<5%
pPB1530-1750	poly-his	no purified protein detected	?
<i>pMB1530-1750</i>	<i>MBP</i>	<i>20mg/l</i>	<i>25%</i>
<i>pMB1750-2360</i>	<i>MBP</i>	<i>>20mg/l</i>	<i>>90%</i>
pMBp1750-2360	MBP	6.5mg/l (secreted)	50%
pPB1750-2360	poly-his	>20mg/l	>90%
pMB1750-1970	MBP	>20mg/l	>90%
pMB1970-2360	MBP	40mg/l	>90%
pMBp1970-2360	MBP	(no secretion)	NA
pMB1850-2360	MBP	20mg/l	>90%
pPB1850-2360	poly-his	15mg/l	>90%
pMB1850-1970	MBP	70mg/l	>90%
pPB1850-1970	poly-his	>10mg/l (insoluble)	>90%
pPB1850-2070	poly-his	>10mg/l (insoluble)	>90%
pPB1750-1970(c)	poly-his	>10mg/l (insoluble)	>90%
pPB1750-1970(n)	poly-his	>10mg/l (insoluble)	>90%

* Clones in italics are clones currently utilized to purify recombinant protein from each selected interval.

EXAMPLE 19

Identification, Purification And Induction Of Neutralizing Antibodies Against Recombinant *C. difficile* Toxin B Protein

To determine whether recombinant toxin B polypeptide fragments can generate neutralizing antibodies, typically animals would first be immunized with recombinant proteins and anti-recombinant antibodies are generated. These anti-recombinant protein antibodies are then tested for neutralizing ability *in vivo* or *in vitro*. Depending on the immunogenic nature of the recombinant polypeptide, the generation of high-titer antibodies against that protein may take several months. To accelerate this process and identify which recombinant polypeptide(s) may be the best candidate to generate neutralizing antibodies, depletion studies were performed. Specifically, recombinant toxin B polypeptide were pre-screened by testing whether they have the ability to bind to protective antibodies from a CTB antibody preparation and hence deplete those antibodies of their neutralizing capacity. Those recombinant polypeptides found to bind CTB, were then utilized to generate neutralizing antibodies. This Example involved: a) identification of recombinant sub-regions within toxin B to which neutralizing antibodies bind; b) identification of toxin B sub-region specific antibodies that neutralize toxin B *in vivo*; and c) generation and evaluation of antibodies reactive to recombinant toxin B polypeptides.

a) Identification Of Recombinant Sub-Regions Within Toxin B To Which Neutralizing Antibodies Bind

Sub-regions within toxin B to which neutralizing antibodies bind were identified by utilizing recombinant toxin B proteins to deplete protective antibodies from a polyclonal pool of antibodies against native *C. difficile* toxin B. An *in vivo* assay was developed to evaluate protein preparations for the ability to bind neutralizing

antibodies. Recombinant proteins were first pre-mixed with antibodies directed against native toxin B (CTB antibody; see Example 8) and allowed to react for one hour at 37°C. Subsequently, *C. difficile* toxin B (CTB; Tech Lab) was added at a concentration lethal to hamsters and incubated for another hour at 37°C. After incubation this mixture was injected intraperitoneally (IP) into hamsters. If the recombinant polypeptide contains neutralizing epitopes, the CTB antibodies will lose its ability to protect the hamsters against death from CTB. If partial or complete protection occurs with the CTB antibody-recombinant mixture, that recombinant contains only weak or non-neutralizing epitopes of toxin B. This assay was performed as follows.

Antibodies against CTB were generated in egg laying Leghorn hens as described in Example 8. The lethal dosage (LD₁₀₀) of *C. difficile* toxin B when delivered I.P. into 40g female Golden Syrian hamsters (Charles River) was determined to be 2.5 to 5 µg. Antibodies generated against CTB and purified by PEG precipitation could completely protect the hamsters at the I.P. dosage determined above. The minimal amount of CTB antibody needed to afford good protection against 5 µg of CTB when injected I.P. into hamsters was also determined (1X PEG prep). These experiments defined the parameters needed to test whether a given recombinant protein could deplete protective CTB antibodies.

The cloned regions tested for neutralizing ability cover the entire toxin B gene and were designated as Intervals (INT) 1 through 5 (see Figure 19). Approximately equivalent final concentrations of each recombinant polypeptide were tested. The following recombinant polypeptides were used: 1) a mixture of intervals 1 and 2 (INT-1, 2); 2) a mixture of Intervals 4 and 5 (INT-4, 5) and 3) Interval 3 (INT-3). Recombinant proteins (each at about 1 mg total protein) were first preincubated with a final CTB antibody concentration of 1X [*i.e.*, pellet dissolved in original yolk volume as described in Example 1(c)] in a final volume of 5 mls for 1 hour at 37°C. Twenty-

five μg of CTB (at a concentration of 5 $\mu\text{g}/\text{ml}$), enough CTB to kill 5 hamsters, was then added and the mixture was then incubated for 1 hour at 37°C. Five, 40g female hamsters (Charles River) in each treatment group were then each given 1 ml of the mixture I.P. using a tuberculin syringe with a 27 gauge needle. The results of this experiment are shown in Table 24.

TABLE 24

Binding Of Neutralizing Antibodies By INT 3 Protein

Treatment Group	Number Of Animals Alive	Number Of Animals Dead
CTB antibodies	3	2
CTB antibodies + INT1,2	3	2
CTB antibodies + INT4,5	3	2
CTB antibodies + INT 3	0	5

C. difficile toxin B (CTB) was added to each group.

As shown in Table 24, the addition of recombinant proteins from INT-1, 2 or INT-4, 5 had no effect on the *in vivo* protective ability of the CTB antibody preparation compared to the CTB antibody preparation alone. In contrast, INT-3 recombinant polypeptide was able to remove all of the toxin B neutralizing ability of the CTB antibodies as demonstrated by the death of all the hamsters in that group.

The above experiment was repeated, using two smaller expressed fragments (pMB 1750-1970 and pMB 1970-2360, see Figure 19) comprising the INT-3 domain to determine if that domain could be further subdivided into smaller neutralizing epitopes. In addition, full-length INT-3 polypeptide expressed as a nickel tagged protein (pPB1750-2360) was tested for neutralizing ability and compared to the original INT-3 expressed MBP fusion (pMB1750-2360). The results are shown in Table 25.

TABLE 25

Removal Of Neutralizing Antibodies By Repeat Containing Proteins

Treatment Group ¹	Number Of Animals Alive	Number Of Animals Dead
CTB antibodies	5	0
CTB antibodies + pPB1750-2360	0	5
CTB antibodies + pMB1750-2360	0	5
CTB antibodies + pMB1970-2360	3	2
CTB antibodies + pMB1750-1970	2	3

¹ *C. difficile* toxin B (CTB) was added to each group.

10 The results summarized in Table 25 indicate that the smaller polypeptide fragments within the INT-3 domain, pMB1750-1970 and pMB1970-2360, partially lose the ability to bind to and remove neutralizing antibodies from the CTB antibody pool. These results demonstrate that the full length INT-3 polypeptide is required to completely deplete the CTB antibody pool of neutralizing antibodies. This experiment also shows that the neutralization epitope of INT-3 can be expressed in alternative vector systems and the results are independent of the vector utilized or the accompanying fusion partner.

Other Interval 3 specific proteins were subsequently tested for the ability to remove neutralizing antibodies within the CTB antibody pool as described above. The Interval 3 specific proteins used in these studies are summarized in Figure 23. In Figure 23 the following abbreviations are used: pP refers to the pET23 vector; pM refers to the pMALc vector; B refers to toxin B; the numbers refer to the amino acid interval expressed in the clone. The solid black ovals represent the MBP; and HHH represents the poly-histidine tag.

25 Only recombinant proteins comprising the entire toxin B repeat domain (pMB1750-2360, pPB1750-2360 and pPB1850-2360) can bind and completely remove neutralizing antibodies from the CTB antibody pool. Recombinant proteins comprising only a portion of the toxin B repeat domain were not capable of completely removing

neutralizing antibodies from the CTB antibody pool (pMB1750-1970 and pMB1970-2360 could partially remove neutralizing antibodies while pMB1850-1970 and pPB1850-2070 failed to remove any neutralizing antibodies from the CTB antibody pool).

5 The above results demonstrate that only the complete ligand binding domain (repeat region) of the toxin B gene can bind and completely remove neutralizing antibodies from the CTB antibody pool. These results demonstrate that antibodies directed against the entire toxin B repeat region are necessary for *in vivo* toxin neutralization (see Figure 23; only the recombinant proteins expressed by the
10 pMB1750-2360, pPB1750-2360 and pPB1850-2360 vectors are capable of completely removing the neutralizing antibodies from the CTB antibody pool).

 These results represent the first indication that the entire repeat region of toxin B would be necessary for the generation of antibodies capable of neutralizing toxin B, and that sub-regions may not be sufficient to generate maximal titers of neutralizing
15 antibodies.

**b) Identification Of Toxin B Sub-Region Specific
 Antibodies That Neutralize Toxin B *In Vivo***

 To determine if antibodies directed against the toxin B repeat region are sufficient for neutralization, region specific antibodies within the CTB antibody
20 preparation were affinity purified, and tested for *in vivo* neutralization. Affinity columns containing recombinant toxin B repeat proteins were made as described below. A separate affinity column was prepared using each of the following recombinant toxin B repeat proteins: pPB1750-2360, pPB1850-2360, pMB1750-1970 and pMB1970-2360.

25 For each affinity column to be made, four ml of PBS-washed Actigel resin (Sterogene) was coupled overnight at room temperature with 5-10 mg of affinity purified recombinant protein (first extensively dialyzed into PBS) in 15 ml tubes (Falcon) containing a 1/10 final volume Ald-coupling solution (1 M sodium cyanoborohydride). Aliquots of the supernatants from the coupling reactions, before

and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based on protein band intensities, in all cases greater than 30% coupling efficiencies were estimated. The resins were poured into 10 ml columns (BioRad), washed extensively with PBS, pre-eluted with 4M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0) and reequilibrated in PBS. The columns were stored at 4°C.

Aliquots of a CTB IgY polyclonal antibody preparation (PEG prep) were affinity purified on each of the four columns as described below. The columns were hooked to a UV monitor (ISCO), washed with PBS and 40 ml aliquots of a 2X PEG prep (filter sterilized using a 0.45 μ filter) were applied. The columns were washed with PBS until the baseline value was re-established. The columns were then washed with BBStween to elute nonspecifically binding antibodies, and reequilibrated with PBS. Bound antibody was eluted from the column in 4M guanidine-HCl (in 10mM Tris-HCl, pH8.0). The eluted antibody was immediately dialyzed against a 100-fold excess of PBS at 4°C for 2 hrs. The samples were then dialyzed extensively against at least 2 changes of PBS, and affinity purified antibody was collected and stored at 4°C. The antibody preparations were quantified by UV absorbance. The elution volumes were in the range of 4-8 ml. All affinity purified stocks contained similar total antibody concentrations, ranging from 0.25-0.35% of the total protein applied to the columns.

The ability of the affinity purified antibody preparations to neutralize toxin B *in vivo* was determined using the assay outlined in a) above. Affinity purified antibody was diluted 1:1 in PBS before testing. The results are shown in Table 26.

In all cases similar levels of toxin neutralization was observed, such that lethality was delayed in all groups relative to preimmune controls. This result demonstrates that antibodies reactive to the repeat region of the toxin B gene are sufficient to neutralize toxin B *in vivo*. The hamsters will eventually die in all groups, but this death is maximally delayed with the CTB PEG prep antibodies. Thus neutralization with the affinity purified (AP) antibodies is not as complete as that observed with the CTB prep before affinity chromatography. This result may be due to loss of activity during guanidine denaturation (during the elution of the antibodies

from the affinity column) or the presence of antibodies specific to other regions of the toxin B gene that can contribute to toxin neutralization (present in the CTB PEG prep).

TABLE 26

Neutralization Of Toxin B By Affinity Purified Antibodies

Treatment group ^a	Number Animals Alive ^b	Number Animals Dead ^b
Preimmune ¹	0	5
CTB ¹ ; 400 µg	5	0
CTB (AP on pPB1750-2360); ² 875 µg	5	0
CTB (AP on pMB1750-1970); ² 875 µg	5	0
CTB (AP on pMB1970-2360); ² 500 µg	5	0

^a *C. difficile* toxin B (CTB) (Tech Lab; at 5 µg/ml, 25 µg total) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as either: ¹4X antibody PEG prep or ²affinity purified (AP) antibody (from CTB PEG prep, on indicated columns). The amount of specific antibody in each prep is indicated; the amount is directly determined for affinity purified preps and is estimated for the 4X CTB as described in Example 15.

^b The numbers in each group represent numbers of hamsters dead or alive, 2 hr post IP administration of toxin/antibody mixture.

The observation that antibodies affinity purified against the non-overlapping pMB1750-1970 and pMB1970-2360 proteins neutralized toxin B raised the possibility that either 1) antibodies specific to repeat sub-regions are sufficient to neutralize toxin B or 2) sub-region specific proteins can bind most or all repeat specific antibodies present in the CTB polyclonal pool. This would likely be due to conformational similarities between repeats, since homology in the primary amino acid sequences between different repeats is in the range of only 25-75% [Eichel-Streiber, *et al.* (1992) Molec. Gen. Genetics 233:260]. These possibilities were tested by affinity chromatography.

The CTB PEG prep was sequentially depleted 2X on the pMB1750-1970 column; only a small elution peak was observed after the second chromatography,

indicating that most reactive antibodies were removed. This interval depleted CTB preparation was then chromatographed on the pPB1850-2360 column; no antibody bound to the column. The reactivity of the CTB and CTB (pMB1750-1970 depleted) preps to pPB1750-2360, pPB1850-2360, pMB1750-1970 and pMB1970-2360 proteins was then determined by ELISA using the protocol described in Example 13(c).
5 Briefly, 96-well microtiter plates (Falcon, Pro-Bind Assay Plates) were coated with recombinant protein by adding 100 μ l volumes of protein at 1-2 μ g/ml in PBS containing 0.005% thimerosal to each well and incubating overnight at 4°C. The next morning, the coating suspensions were decanted and the wells were washed three times
10 using PBS. In order to block non-specific binding sites, 100 μ l of 1.0% BSA (Sigma) in PBS (blocking solution) was then added to each well, and the plates were incubated for 1 hr. at 37°C. The blocking solution was decanted and duplicate samples of 150 μ l of diluted antibody was added to the first well of a dilution series. The initial testing serum dilution was (1/200 for CTB prep, (the concentration of depleted CTB was standardized by OD₂₈₀) in blocking solution containing 0.5% Tween 20, followed
15 by 5-fold serial dilutions into this solution. This was accomplished by serially transferring 30 μ l aliquots to 120 μ l buffer, mixing, and repeating the dilution into a fresh well. After the final dilution, 30 μ l was removed from the well such that all wells contained 120 μ l final volume. A total of 5 such dilutions were performed (4
20 wells total). The plates were incubated for 1 hr at 37°C. Following this incubation, the serially diluted samples were decanted and the wells were washed three times using PBS containing 0.5% Tween 20 (PBST), followed by two 5 min washes using BBS-Tween and a final three washes using PBST. To each well, 100 μ l of 1/1000 diluted secondary antibody [rabbit anti-chicken IgG alkaline phosphatase (Sigma) diluted in
25 blocking solution containing 0.5% Tween 20] was added, and the plate was incubated 1 hr at 37°C. The conjugate solutions were decanted and the plates were washed 6 times in PBST, then once in 50 mM Na₂CO₃, 10 mM MgCl₂, pH 9.5. The plates were developed by the addition of 100 μ l of a solution containing 1 mg/ml para-nitro phenyl phosphate (Sigma) dissolved in 50 mM Na₂CO₃, 10 mM MgCl₂, pH9.5 to each
30 well. The plates were then incubated at room temperature in the dark for 5-45 min.

The absorbency of each well was measured at 410 nm using a Dynatech MR 700 plate reader.

As predicted by the affinity chromatography results, depletion of the CTB prep on the pMB1750-1970 column removed all detectable reactivity to the pMB1970-2360 protein. The reciprocal purification of a CTB prep that was depleted on the pMB1970-2360 column yielded no bound antibody when chromatographed on the pMB1750-1970 column. These results demonstrate that all repeat reactive antibodies in the CTB polyclonal pool recognize a conserved structure that is present in non-overlapping repeats. Although it is possible that this conserved structure represents rare conserved linear epitopes, it appears more likely that the neutralizing antibodies recognize a specific protein conformation. This conclusion was also suggested by the results of Western blot hybridization analysis of CTB reactivity to these recombinant proteins.

Western blots of 7.5% SDS-PAGE gels, loaded and electrophoresed with defined quantities of each recombinant protein, were probed with the CTB polyclonal antibody preparation. The blots were prepared and developed with alkaline phosphatase as described in Example 3. The results are shown in Figure 24.

Figure 24 depicts a comparison of immunoreactivity of IgY antibody raised against either native or recombinant toxin B antigen. Equal amounts of pMB1750-1970 (lane 1), pMB1970-2360 (lane 2), pPB1850-2360 (lane 3) as well as a serial dilution of pPB1750-2360 (lanes 4-6 comprising 1X, 1/10X and 1/100X amounts, respectively) proteins were loaded in duplicate and resolved on a 7.5% SDS-PAGE gel. The gel was blotted and each half was hybridized with PEG prep IgY antibodies from chickens immunized with either native CTB or pPB1750-2360 protein. Note that the full-length pMB1750-1970 protein was identified only by antibodies reactive to the recombinant protein (arrows).

Although the CTB prep reacts with the pPB1750-2360, pPB1850-2360, and pMB1970-2360 proteins, no reactivity to the pMB1750-1970 protein was observed (Figure 24). Given that all repeat reactive antibodies can be bound by this protein during affinity chromatography, this result indicates that the protein cannot fold

properly on Western blots. Since this eliminates all antibody reactivity, it is unlikely that the repeat reactive antibodies in the CTB prep recognize linear epitopes. This may indicate that in order to induce protective antibodies, recombinant toxin B protein will need to be properly folded.

5 c) **Generation And Evaluation Of Antibodies Reactive To
Recombinant Toxin B Polypeptides**

 i) **Generation Of Antibodies Reactive To
Recombinant Toxin B Proteins**

10 Antibodies against recombinant proteins were generated in egg laying Leghorn hens as
described in Example 13. Antibodies were raised [using Freund's adjuvant (Gibco)
unless otherwise indicated] against the following recombinant proteins: 1) a mixture
of Interval 1+2 proteins (see Figure 18); 2) a mixture of interval 4 and 5 proteins (see
Figure 18); 3) pMB1970-2360 protein; 4) pPB1750-2360 protein; 5) pMB1750-2360;
6) pMB1750-2360 [Titermax adjuvant (Vaxcell)]; 7) pMB1750-2360 [Gerbu adjuvant
15 (Biotech)]; 8) pMBp1750-2360 protein; 9) pPB1850-2360; and 10) pMB1850-2360.

 Chickens were boosted at least 3 times with recombinant protein until ELISA
reactivity [using the protocol described in b) above with the exception that the plates
were coated with pPB1750-2360 protein] of polyclonal PEG preps was at least equal
to that of the CTB polyclonal antibody PEG prep. ELISA titers were determined for
20 the PEG preps from all of the above immunogens and were found to be comparable
ranging from 1:12500 to 1:62500. High titers were achieved in all cases except in 6)
pMB1750-2360 in which strong titers were not observed using the Titermax adjuvant,
and this preparation was not tested further.

**ii) Evaluation Of Antibodies Reactive To
Recombinant Proteins By Western Blot
Hybridization**

Western blots of 7.5% SDS-PAGE gels, loaded and electrophoresed with defined quantities of recombinant protein (pMB1750-1970, pPB1850-2360, and pMB1970-2360 proteins and a serial dilution of the pPB1750-2360 to allow quantification of reactivity), were probed with the CTB, pPB1750-2360, pMB1750-2360 and pMB1970-2360 polyclonal antibody preparations (from chickens immunized using Freund's adjuvant). The blots were prepared and developed with alkaline phosphatase as described above in b).

As shown in Figure 24, the CTB and pMB1970-2360 preps reacted strongly with the pPB1750-2360, pPB1850-2360, and pMB1970-2360 proteins while the pPB1750-2360 and pMB1970-2360 (Gerbu) preparations reacted strongly with all four proteins. The Western blot reactivity of the pPB1750-2360 and pMB1970-2360 (Gerbu) preparations were equivalent to that of the CTB preparation, while reactivity of the pMB1970-2360 preparation was <10% that of the CTB prep. Despite equivalent ELISA reactivities only weak reactivity (approximately 1%) to the recombinant proteins were observed in PEG preps from two independent groups immunized with the pMB1750-2360 protein and one group immunized with the pMB1750-2360 preparation using Freund's adjuvant.

Affinity purification was utilized to determine if this difference in immunoreactivity by Western blot analysis reflects differing antibody titers. Fifty ml 2X PEG preparations from chickens immunized with either pMB1750-2360 or pMB1970-2360 protein were chromatographed on the pPB1750-2360 affinity column from b) above, as described. The yield of affinity purified antibody (% total protein in preparation) was equivalent to the yield obtained from a CTB PEG preparation in b) above. Thus, differences in Western reactivity reflect a qualitative difference in the antibody pools, rather than quantitative differences. These results demonstrate that certain recombinant proteins are more effective at generating high affinity antibodies (as assayed by Western blot hybridization).

iii) ***In Vivo* Neutralization Of Toxin B Using
Antibodies Reactive To Recombinant
Protein**

5 The *in vivo* hamster model [described in Examples 9 and 14(b)] was utilized to assess the neutralizing ability of antibodies raised against recombinant toxin B proteins. The results from three experiments are shown below in Tables 27-29.

10 The ability of each immunogen to neutralize toxin B *in vivo* has been compiled and is shown in Table 30. As predicted from the recombinant protein-CTB premix studies (Table 24) only antibodies to Interval 3 (1750-2366) and not the other regions of toxin B (*i.e.*, intervals 1-5) are protective. Unexpectedly, antibodies generated to INT-3 region expressed in pMAL vector (pMB1750-2360 and pMpB1750-2360) using
15 Freund's adjuvant were non-neutralizing. This observation is reproducible, since no neutralization was observed in two independent immunizations with pMB1750-2360 and one immunization with pMpB1750-2360. The fact that 5X quantities of affinity purified toxin B repeat specific antibodies from pMB1750-2360 PEG preps cannot neutralize toxin B while 1X quantities of affinity purified anti-CTB antibodies can (Table 28) demonstrates that the differential ability of CTB antibodies to neutralize toxin B is due to qualitative rather than quantitative differences in these antibody
20 preparations. Only when this region was expressed in an alternative vector (pPB1750-2360) or using an alternative adjuvant with the pMB1750-2360 protein were neutralizing antibodies generated. Importantly, antibodies raised using Freund's adjuvant to pPB1850-2360, which contains a fragment that is only 100 amino acids smaller than recombinant pPB1750-2360, are unable to neutralize toxin B *in vivo* (Table 27); note also that the same vector is used for both pPB1850-2360 and
25 pPB1750-2360.

TABLE 27

In Vivo Neutralization Of Toxin B

Treatment Group ^a	Number Animals Alive ^b	Number Animals Dead ^b
Preimmune	0	5
CTB	5	0
INT1+2	0	5
INT 4+5	0	5
pMB1750-2360	0	5
pMB1970-2360	0	5
pPB1750-2360	5	0

^a *C. difficile* toxin B (CTB) (at 5 µg/ml; 25 µg total; Tech Lab) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as a 4X antibody PEG prep.

^b The numbers in each group represent numbers of hamsters dead or alive, 2 hours post IP administration of toxin/antibody mixture.

TABLE 28

In Vivo Neutralization Of Toxin B Using Affinity Purified Antibodies

Treatment Group ^a	Number Animals Alive ^b	Number Animals Dead ^b
Preimmune(1)	0	5
CTB(1)	5	0
pPB1750-2360(1)	5	0
1.5 mg anti-pMB1750-2360(2)	1	4
1.5 mg anti-pMB1970-2360(2)	0	5
300 µg anti-CTB(2)	5	0

^a *C. difficile* toxin B (CTB) (at 5 µg/ml; 25 µg total; Tech Lab) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation, 1 ml of this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as either (1) 4X antibody PEG prep or (2) affinity purified antibody (on a pPB1750-2360 resin), either 1.5 mg/group (anti-pMB1750-2360 and anti-pMB1970-2360; used undiluted affinity purified antibody) or 350 µg/group (anti-CTB, repeat specific; used 1/5 diluted anti-CTB antibody).

^b The numbers in each group represent numbers of hamsters dead or alive, 2 hr post-IP administration of toxin/antibody mixture.

TABLE 29

Generation Of Neutralizing Antibodies Utilizing The Gerbu Adjuvant

Treatment Group ^a	Number Animals Alive ^b	Number Animals Dead ^b
Preimmune	0	5
CTB	5	0
pMB1970-2360	0	5
pMB1850-2360	0	5
pPB1850-2360	0	5
pMB1750-2360 (Gerbu adj)	5	0

^a *C. difficile* toxin B (CTB) (Tech Lab) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as a 4X antibody PEG prep.

^b The numbers in each group represent numbers of hamsters dead or alive, 2hrs post IP administration of toxin/antibody mixture.

TABLE 30

In Vivo Neutralization Of Toxin B

Immunogen	Adjuvant	Tested Preparation ^a	Antigen Utilized For AP	<i>In vivo</i> Neutralization ^b
Preimmune	NA ^c	PEG	NA	no
CTB (native)	Titermax	PEG	NA	yes
CTB (native)	Titermax	AP	pPB1750-2360	yes
CTB (native)	Titermax	AP	pPB1850-2360	yes
CTB (native)	Titermax	AP	pPB1750-1970	yes
CTB (native)	Titermax	AP	pPB1970-2360	yes
pMB1750-2360	Freunds	PEG	NA	no
pMB1750-2360	Freunds	AP	pPB1750-2360	no
pMB1750-2360	Gerbu	PEG	NA	yes
pMB1970-2360	Freunds	PEG	NA	no
pMB1970-2360	Freunds	AP	pPB1750-2360	no
pPB1750-2360	Freunds	PEG	NA	yes
pPB1850-2360	Freunds	PEG	NA	no
pMB1850-2360	Freunds	PEG	NA	no
INT 1+2	Freunds	PEG	NA	no
INT 4+5	Freunds	PEG	NA	no

^a Either PEG preparation (PEG) or affinity purified antibodies (AP).

^b 'Yes' denotes complete neutralization (0/5 dead) while 'no' denotes no neutralization (5/5 dead) of toxin B, 2 hours post-administration of mixture.

^c 'NA' denotes not applicable.

The pPB1750-2360 antibody pool confers significant *in vivo* protection, equivalent to that obtained with the affinity purified CTB antibodies. This correlates with the observed high affinity of this antibody pool (relative to the pMB1750-2360 or pMB1970-2360 pools) as assayed by Western blot analysis (Figure 24). These results provide the first demonstration that *in vivo* neutralizing antibodies can be induced using recombinant toxin B protein as immunogen.

The failure of high concentrations of antibodies raised against the pMB1750-2360 protein (using Freund's adjuvant) to neutralize, while the use of Gerbu adjuvant and pMB1750-2360 protein generates a neutralizing response, demonstrates that conformation or presentation of this protein is essential for the induction of neutralizing antibodies. These results are consistent with the observation that the neutralizing antibodies produced when native CTB is used as an immunogen appear to recognize conformational epitopes [see section b) above]. This is the first demonstration that the conformation or presentation of recombinant toxin B protein is essential to generate high titers of neutralizing antibodies.

EXAMPLE 20

Determination Of Quantitative And Qualitative
Differences Between pMB1750-2360, pMB1750-2360 (Gerbu)
Or pPB1750-2360 IgY Polyclonal Antibody Preparations

In Example 19, it was demonstrated that toxin B neutralizing antibodies could be generated using specific recombinant toxin B proteins (pPB1750-2360) or specific adjuvants. Antibodies raised against pMB1750-2360 were capable of neutralizing the enterotoxin effect of toxin B when the recombinant protein was used to immunize hens in conjunction with the Gerbu adjuvant, but not when Freund's adjuvant was used. To determine the basis for these antigen and adjuvant restrictions, toxin B-specific antibodies present in the neutralizing and non-neutralizing PEG preparations were isolated by affinity chromatography and tested for qualitative or quantitative differences. The example involved a) purification of anti-toxin B specific antibodies from pMB1750-2360 and pPB1750-2360 PEG preparations and b) *in vivo* neutralization of toxin B using the affinity purified antibody.

a) **Purification Of specific Antibodies From
pMB1750-2360 And pPB1750-2360 PEG Preparations**

To purify and determine the concentration of specific antibodies (expressed as the percent of total antibody) within the pPB1750-2360 (Freunds and Gerbu) and pPB1750-2360 PEG preparations, defined quantities of these antibody preparations were chromatographed on an affinity column containing the entire toxin B repeat region (pPB1750-2360). The amount of affinity purified antibody was then quantified.

An affinity column containing the recombinant toxin B repeat protein, pPB1750-2360, was made as follows. Four ml of PBS-washed Actigel resin (Sterogene) was coupled with 5 mg of pPB1750-2360 affinity purified protein (dialyzed into PBS; estimated to be greater than 95% full length fusion protein) in a 15 ml tube (Falcon) containing 1/10 final volume Ald-coupling solution (1M sodium cyanoborohydride). Aliquots of the supernatant from the coupling reactions, before and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based on protein band intensities, greater than 95% (approximately 5 mg) of recombinant protein was coupled to the resin. The coupled resin was poured into a 10 ml column (BioRad), washed extensively with PBS, pre-eluted with 4M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0; 0.005% thimerosal) and re-equilibrated in PBS and stored at 4°C.

Aliquots of pMB1750-2360, pMB1750-2360 (Gerbu) or pPB1750-2360 IgY polyclonal antibody preparations (PEG preps) were affinity purified on the above column as follows. The column was attached to an UV monitor (ISCO), and washed with PBS. Forty ml aliquots of 2X PEG preps (filter sterilized using a 0.45 μ filter and quantified by OD₂₈₀ before chromatography) was applied. The column was washed with PBS until the baseline was re-established (the column flow-through was saved), washed with BBSTween to elute nonspecifically binding antibodies and re-equilibrated with PBS. Bound antibody was eluted from the column in 4M guanidine-HCl (in 10 mM Tris-HCL, pH 8.0, 0.005% thimerosal) and the entire elution peak collected in a 15 ml tube (Falcon). The column was re-equilibrated, and the column eluate re-chromatographed as described above. The antibody preparations were

quantified by UV absorbance (the elution buffer was used to zero the spectrophotometer). Approximately 10 fold higher concentrations of total purified antibody was obtained upon elution of the first chromatography pass relative to the second pass. The low yield from the second chromatography pass indicated that most of the specific antibodies were removed by the first round of chromatography.

Pools of affinity purified specific antibodies were prepared by dialysis of the column elutes after the first column chromatography pass for the pMB1750-2360, pMB1750-2360 (Gerbu) or pPB1750-2360 IgY polyclonal antibody preparations. The elutes were collected on ice and immediately dialyzed against a 100-fold volume of PBS at 4°C for 2 hrs. The samples were then dialyzed against 3 changes of a 65-fold volume of PBS at 4°C. Dialysis was performed for a minimum of 8 hrs per change of PBS. The dialyzed samples were collected, centrifuged to remove insoluble debris, quantified by OD₂₈₀, and stored at 4°C.

The percentage of toxin B repeat-specific antibodies present in each preparation was determined using the quantifications of antibody yields from the first column pass (amount of specific antibody recovered after first pass/total protein loaded). The yield of repeat-specific affinity purified antibody (expressed as the percent of total protein in the preparation) in: 1) the pMB1750-2360 PEG prep was approximately 0.5%, 2) the pMB1750-2360 (Gerbu) prep was approximately 2.3%, and 3) the pPB1750-2360 prep was approximately 0.4%. Purification of a CTB IgY polyclonal antibody preparation on the same column demonstrated that the concentration of toxin B repeat specific antibodies in the CTB preparation was 0.35%.

These results demonstrate that 1) the use of Gerbu adjuvant enhanced the titer of specific antibody produced against the pMB1750-2360 protein 5-fold relative to immunization using Freund's adjuvant, and 2) the differences seen in the *in vivo* neutralization ability of the pMB1750-2360 (not neutralizing) and pPB1750-2360 (neutralizing) and CTB (neutralizing) PEG preps seen in Example 19 was not due to differences in the titers of repeat-specific antibodies in the three preparations because the titer of repeat-specific antibody was similar for all three preps; therefore the differing ability of the three antibody preparations to neutralize toxin B must reflect

qualitative differences in the induced toxin B repeat-specific antibodies. To confirm that qualitative differences exist between antibodies raised in hens immunized with different recombinant proteins and/or different adjuvants, the same amount of affinity purified anti-toxin B repeat (aa 1870-2360 of toxin B) antibodies from the different preparations was administered to hamsters using the *in vivo* hamster model as described below.

b) *In vivo* Neutralization Of Toxin B Using Affinity Purified Antibody

The *in vivo* hamster model was utilized to assess the neutralizing ability of the affinity purified antibodies raised against recombinant toxin B proteins purified in (a) above. As well, a 4X IgY PEG preparation from a second independent immunization utilizing the pPB1750-2360 antigen with Freund's adjuvant was tested for *in vivo* neutralization. The results are shown in Table 31.

The results shown in Table 31 demonstrate that:

- 1) as shown in Example 19 and reproduced here, 1.5 mg of affinity purified antibody from pMB1750-2360 immunized hens using Freund's adjuvant does not neutralize toxin B *in vivo*. However, 300 µg of affinity purified antibody from similarly immunized hens utilizing Gerbu adjuvant demonstrated complete neutralization of toxin B *in vivo*. This demonstrates that Gerbu adjuvant, in addition to enhancing the titer of antibodies reactive to the pMB1750-2360 antigen relative to Freund's adjuvant (demonstrated in (a) above), also enhances the yield of neutralizing antibodies to this antigen, greater than 5 fold.
- 2) Complete *in vivo* neutralization of toxin B was observed with 1.5 mg of affinity purified antibody from hens immunized with pPB1750-2360 antigen, but not with pMB1750-2360 antigen, when Freund's adjuvant was used. This demonstrates, using standardized toxin B repeat-specific antibody concentrations, that neutralizing antibodies were induced when

pPB1750-2360 but not pMB1750-2360 was used as the antigen with Freund's adjuvant.

- 3) Complete *in vivo* neutralization was observed with 300 µg of pMB1750-2360 (Gerbu) antibody, but not with 300 µg of pPB1750-2360 (Freund's) antibody. Thus the pMB1750-2360 (Gerbu) antibody has a higher titer of neutralizing antibodies than the pPB1750-2360 (Freund's) antibody.
- 4) Complete neutralization of toxin B was observed using 300 µg of CTB antibody [affinity purified (AP)] but not 100 µg CTB antibody (AP or PEG prep). This demonstrates that greater than 100 µg of toxin B repeat-specific antibody (anti-CTB) is necessary to neutralize 25 µg toxin B *in vivo* in this assay, and that affinity purified antibodies specific to the toxin B repeat interval neutralize toxin B as effectively as the PEG prep of IgY raised against the entire CTB protein (shown in this assay).
- 5) As was observed with the initial pPB1750-2360 (IgY) PEG preparation (Example 19), complete neutralization was observed with a IgY PEG preparation isolated from a second independent group of pPB1750-2360 (Freund's) immunized hens. This demonstrates that neutralizing antibodies are reproducibly produced when hens are immunized with pPB1750-2360 protein utilizing Freund's adjuvant.

TABLE 31

In vivo Neutralization Of Toxin B Using Affinity Purified Antibodies

Treatment Group ^a	Number Animals Alive ^b	Number Animals Dead ^b
Preimmune ¹	0	5
CTB (300 µg) ²	5	0
CTB (100 µg) ²	1	4
pMB1750-2360 (G) (5 mg) ²	5	0
pMB1750-2360 (G) (1.5 mg) ²	5	0
pMB1750-2360 (G) (300 µg) ²	5	0
pMB1750-2360 (F) (1.5 mg) ²	0	5
pPB1750-2360 (F) (1.5 mg) ²	5	0
pPB1750-2360 (F) (300 µg) ²	1	4
CTB (100 µg) ³	2	3
pPB1750-2360 (F) (500 µg) ¹	5	0

C. difficile toxin B (CTB) (Tech Lab) at lethal concentration to hamsters (25 µg) was added to the antibody (amount of specific antibody is indicated) and incubated for one hour at 37°C. After incubation, this mixture was injected IP into hamsters (1/5 total mix injected per hamster). Each treatment group received toxin premixed with antibody raised against the indicated protein (G=gerbu adjuvant, F=Freunds adjuvant). ¹ indicates the antibody was a 4X IgY PEG prep; ² indicates the antibody was affinity purified on a pPB1850-2360 resin; and ³ indicates that the antibody was a 1X IgY PEG prep.

The numbers in each group represent numbers of hamsters dead or alive, 2 hrs post IP administration of toxin/antibody mixture.

EXAMPLE 21

Diagnostic Enzyme Immunoassays For *C. difficile* Toxins A And B

The ability of the recombinant toxin proteins and antibodies raised against these recombinant proteins (described in the above examples) to form the basis of diagnostic assays for the detection of clostridial toxin in a sample was examined. Two immunoassay formats were tested to quantitatively detect *C. difficile* toxin A and toxin B from a biological specimen. The first format involved a competitive assay in which

a fixed amount of recombinant toxin A or B was immobilized on a solid support (e.g., microtiter plate wells) followed by the addition of a toxin-containing biological specimen mixed with affinity-purified or PEG fractionated antibodies against recombinant toxin A or B. If toxin is present in a specimen, this toxin will compete with the immobilized recombinant toxin protein for binding to the anti-recombinant antibody thereby reducing the signal obtained following the addition of a reporter reagent. The reporter reagent detects the presence of antibody bound to the immobilized toxin protein.

In the second format, a sandwich immunoassay was developed using affinity-purified antibodies to recombinant toxin A and B. The affinity-purified antibodies to recombinant toxin A and B were used to coat microtiter wells instead of the recombinant polypeptides (as was done in the competitive assay format). Biological samples containing toxin A or B were then added to the wells followed by the addition of a reporter reagent to detect the presence of bound toxin in the well.

a) **Competitive Immunoassay For The Detection Of
C. difficile Toxin**

Recombinant toxin A or B was attached to a solid support by coating 96 well microtiter plates with the toxin protein at a concentration of 1 µg/ml in PBS. The plates were incubated overnight at 2-8°C. The following morning, the coating solutions were removed and the remaining protein binding sites on the wells were blocked by filling each well with a PBS solution containing 0.5% BSA and 0.05% Tween-20. Native *C. difficile* toxin A or B (Tech Lab) was diluted to 4 µg/ml in stool extracts from healthy Syrian hamsters (Sasco). The stool extracts were made by placing fecal pellets in a 15 ml centrifuge tube; PBS was added at 2 ml/pellet and the tube was vortexed to create a uniform suspension. The tube was then centrifuged at 2000 rpm for 5 min at room temperature. The supernatant was removed; this comprises the stool extract. Fifty µl of the hamster stool extract was pipetted into each well of the microtiter plates to serve as the diluent for serial dilutions of the 4 µg/ml toxin samples. One hundred µl of the toxin samples at 4 µg/ml was pipetted

into the first row of wells in the microtiter plate, and 50 μ l aliquots were removed and diluted serially down the plate in duplicate. An equal volume of affinity purified anti-recombinant toxin antibodies [1 ng/well of anti-pMA1870-2680 antibody was used for the detection of toxin A; 0.5 ng/well of anti-pMB1750-2360(Gerbu) was used for the detection of toxin B] were added to appropriate wells, and the plates were incubated at room temperature for 2 hours with gentle agitation. Wells serving as negative control contained antibody but no native toxin to compete for binding.

Unbound toxin and antibody were removed by washing the plates 3 to 5 times with PBS containing 0.05% Tween-20. Following the wash step, 100 μ l of rabbit anti-chicken IgG antibody conjugated to alkaline phosphatase (Sigma) was added to each well and the plates were incubated for 2 hours at room temperature. The plates were then washed as before to remove unbound secondary antibody. Freshly prepared alkaline phosphatase substrate (1 mg/ml p-nitrophenyl phosphate (Sigma) in 50 mM Na_2CO_3 , pH 9.5; 10 mM MgCl_2) was added to each well. Once sufficient color developed, the plates were read on a Dynatech MR700 microtiter plate reader using a 410 nm filter.

The results are summarized in Tables 32 and 33. For the results shown in Table 32, the wells were coated with recombinant toxin A protein (pMA1870-2680). The amount of native toxin A added (present as an addition to solubilized hamster stool) to a given well is indicated (0 to 200 ng). Antibody raised against the recombinant toxin A protein, pMA1870-2680, was affinity purified on the an affinity column containing pPA1870-2680 (described in Example 20). As shown in Table 32, the recombinant toxin A protein and affinity-purified antitoxin can be used for the basis of a competitive immunoassay for the detection of toxin A in biological samples.

Similar results were obtained using the recombinant toxin B, pPB1750-2360, and antibodies raised against pMB1750-2360(Gerbu). For the results shown in Table 33, the wells were coated with recombinant toxin B protein (pPB1750-2360). The amount of native toxin B added (present as an addition to solubilized hamster stool) to a given well is indicated (0 to 200 ng). Antibody raised against the recombinant toxin B protein, pMB1750-2360(Gerbu), was affinity purified on the an

affinity column containing pPB1850-2360 (described in Example 20). As shown in Table 33, the recombinant toxin B protein and affinity-purified antitoxin can be used for the basis of a competitive immunoassay for the detection of toxin B in biological samples.

- 5 In this competition assay, the reduction is considered significant over the background levels at all points; therefore the assay can be used to detect samples containing less than 12.5 ng toxin A/well and as little as 50-100 ng toxin B/well.

TABLE 32
Competitive Inhibition Of Anti-*C. difficile* Toxin A By Native Toxin A

10

ng Toxin A/Well	OD ₄₁₀ Readout	
200	0.176	
100	0.253	
50	0.240	
25	0.259	
15	12.5	0.309
6.25	0.367	
3.125	0.417	
0	0.590	

TABLE 33

Competitive Inhibition Of Anti-*C. difficile* Toxin B By Native Toxin B

ng Toxin B/Well	OD ₄₁₀ Readout
200	0.392
100	0.566
50	0.607
25	0.778
12.5	0.970
6.25	0.902
3.125	1.040
0	1.055

These competitive inhibition assays demonstrate that native *C. difficile* toxins and recombinant *C. difficile* toxin proteins can compete for binding to antibodies raised against recombinant *C. difficile* toxins demonstrating that these anti-recombinant toxin antibodies provide effective diagnostic reagents.

**b) Sandwich Immunoassay For The Detection Of
C. difficile Toxin**

Affinity-purified antibodies against recombinant toxin A or toxin B were immobilized to 96 well microtiter plates as follows. The wells were passively coated overnight at 4°C with affinity purified antibodies raised against either pMA1870-2680 (toxin A) or pMB1750-2360(Gerbu) (toxin B). The antibodies were affinity purified as described in Example 20. The antibodies were used at a concentration of 1 µg/ml and 100 µl was added to each microtiter well. The wells were then blocked with 200 µl of 0.5% BSA in PBS for 2 hours at room temperature and the blocking solution was then decanted. Stool samples from healthy Syrian hamsters were resuspended in PBS, pH 7.4 (2 ml PBS/stool pellet was used to resuspend the pellets and the sample was centrifuged as described above). The stool suspension was then spiked with native *C. difficile* toxin A or B (Tech Lab) at 4 µg/ml. The stool suspensions

containing toxin (either toxin A or toxin B) were then serially diluted two-fold in stool suspension without toxin and 50 µl was added in duplicate to the coated microtiter wells. Wells containing stool suspension without toxin served as the negative control.

5 The plates were incubated for 2 hours at room temperature and then were washed three times with PBS. One hundred µl of either goat anti-native toxin A or goat anti-native toxin B (Tech Lab) diluted 1:1000 in PBS containing 1% BSA and 0.05% Tween 20 was added to each well. The plates were incubated for another 2 hours at room temperature. The plates were then washed as before and 100 µl of alkaline phosphatase-conjugated rabbit anti-goat IgG (Cappel, Durham, N.C.) was
10 added at a dilution of 1:1000. The plates were incubated for another 2 hours at room temperature. The plates were washed as before then developed by the addition of 100 µl/well of a substrate solution containing 1 mg/ml p-nitrophenyl phosphate (Sigma) in 50 mM Na₂CO₃, pH 9.5; 10 mM MgCl₂. The absorbance of each well was measured using a plate reader (Dynatech) at 410 nm. The assay results are shown in Tables 34
15 and 35.

TABLE 34

C. difficile Toxin A Detection In Stool Using Affinity-Purified Antibodies Against Toxin A

ng Toxin A/Well	OD ₄₁₀ Readout
200	0.9
100	0.8
50	0.73
25	0.71
12.5	0.59
6.25	0.421
0	0

TABLE 35

C. difficile Toxin B Detection In Stool Using Affinity-Purified Antibodies Against Toxin B

ng Toxin B/Well	OD ₄₁₀ Readout
200	1.2
100	0.973
50	0.887
25	0.846
12.5	0.651
6.25	0.431
0	0.004

The results shown in Tables 34 and 35 show that antibodies raised against recombinant toxin A and toxin B fragments can be used to detect the presence of *C. difficile* toxin in stool samples. These antibodies form the basis for a sensitive sandwich immunoassay which is capable of detecting as little as 6.25 ng of either toxin A or B in a 50 μ l stool sample. As shown above in Tables 34 and 35, the background for this sandwich immunoassay is extremely low; therefore, the sensitivity of this assay is much lower than 6.25 ng toxin/well. It is likely that toxin levels of 0.5 to 1.0 pg/well could be detected by this assay.

The results shown above in Tables 32-35 demonstrate clear utility of the recombinant reagents in *C. difficile* toxin detection systems.

EXAMPLE 22

Construction And Expression Of *C. botulinum* C Fragment Fusion Proteins

The *C. botulinum* type A neurotoxin gene has been cloned and sequenced [Thompson, *et al.*, Eur. J. Biochem. 189:73 (1990)]. The nucleotide sequence of the toxin gene is available from the EMBL/GenBank sequence data banks under the accession number X52066; the nucleotide sequence of the coding region is listed in SEQ ID NO:27. The amino acid sequence of the *C. botulinum* type A neurotoxin is

listed in SEQ ID NO:28. The type A neurotoxin gene is synthesized as a single polypeptide chain which is processed to form a dimer composed of a light and a heavy chain linked via disulfide bonds. The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H_C domain.

5 Previous attempts by others to express polypeptides comprising the C fragment of *C. botulinum* type A toxin as a native polypeptide (e.g., not as a fusion protein) in *E. coli* have been unsuccessful [H.F. LaPenotiere, *et al.* in *Botulinum and Tetanus Neurotoxins*, DasGupta, Ed., Plenum Press, New York (1993), pp. 463-466].

10 Expression of the C fragment as a fusion with the *E. coli* MBP was reported to result in the production of insoluble protein (H.F. LaPenotiere, *et al.*, *supra*).

 In order to produce soluble recombinant C fragment proteins in *E. coli*, fusion proteins comprising a synthetic C fragment gene derived from the *C. botulinum* type A toxin and either a portion of the *C. difficile* toxin protein or the MBP were constructed. This example involved a) the construction of plasmids encoding C
15 fragment fusion proteins and b) expression of *C. botulinum* C fragment fusion proteins in *E. coli*.

a) **Construction Of Plasmids Encoding C Fragment
 Fusion Proteins**

 In Example 11, it was demonstrated that the *C. difficile* toxin A repeat domain
20 can be efficiently expressed and purified in *E. coli* as either native (expressed in the pET 23a vector in clone pPA1870-2680) or fusion (expressed in the pMALc vector as a fusion with the *E. coli* MBP in clone pMA1870-2680) proteins. Fusion proteins comprising a fusion between the MBP, portions of the *C. difficile* toxin A repeat domain (shown to be expressed as a soluble fusion protein) and the C fragment of the
25 *C. botulinum* type A toxin were constructed. A fusion protein comprising the C fragment of the *C. botulinum* type A toxin and the MBP was also constructed.

 Figure 25 provides a schematic representation of the botulinal fusion proteins along with the donor constructs containing the *C. difficile* toxin A sequences or *C.*

botulinum C fragment sequences which were used to generate the botulinal fusion proteins. In Figure 25, the solid boxes represent *C. difficile* toxin A gene sequences, the open boxes represent *C. botulinum* C fragment sequences and the solid black ovals represent the *E. coli* MBP. When the name for a restriction enzyme appears inside parenthesis, this indicates that the restriction site was destroyed during construction. An asterisk appearing with the name for a restriction enzyme indicates that this restriction site was recreated at the cloning junction.

In Figure 25, a restriction map of the pMA1870-2680 and pPA1100-2680 constructs (described in Example 11) which contain sequences derived from the *C. difficile* toxin A repeat domain are shown; these constructs were used as the source of *C. difficile* toxin A gene sequences for the construction of plasmids encoding fusions between the *C. botulinum* C fragment gene and the *C. difficile* toxin A gene. The pMA1870-2680 expression construct expresses high levels of soluble, intact fusion protein (20 mg/liter culture) which can be affinity purified on an amylose column (purification described in Example 11d).

The pAlterBot construct (Figure 25) was used as the source of *C. botulinum* C fragment gene sequences for the botulinal fusion proteins. pAlterBot was obtained from J. Middlebrook and R. Lemley at the U.S. Department of Defense. pAlterBot contains a synthetic *C. botulinum* C fragment inserted in to the pALTER-1® vector (Promega). This synthetic C fragment gene encodes the same amino acids as does the naturally occurring C fragment gene. The naturally occurring C fragment sequences, like most clostridial genes, are extremely A/T rich (Thompson *et al.*, *supra*). This high A/T content creates expression difficulties in *E. coli* and yeast due to altered codon usage frequency and fortuitous polyadenylation sites, respectively. In order to improve the expression of C fragment proteins in *E. coli*, a synthetic version of the gene was created in which the non-preferred codons were replaced with preferred codons.

The nucleotide sequence of the *C. botulinum* C fragment gene sequences contained within pAlterBot is listed in SEQ ID NO:22. The first six nucleotides

(ATGGCT) encode a methionine and alanine residue, respectively. These two amino acids result from the insertion of the *C. botulinum* C fragment sequences into the pALTER® vector and provide the initiator methionine residue. The amino acid sequence of the *C. botulinum* C fragment encoded by the sequences contained within pAlterBot is listed in SEQ ID NO:23. The first two amino acids (Met Ala) are encoded by vector-derived sequences. From the third amino acid residue onward (Arg), the amino acid sequence is identical to that found in the *C. botulinum* type A toxin gene.

The pMA1870-2680, pPA1100-2680 and pAlterBot constructs were used as progenitor plasmids to make expression constructs in which fragments of the *C. difficile* toxin A repeat domain were expressed as genetic fusions with the *C. botulinum* C fragment gene using the pMAL-c expression vector (New England BioLabs). The pMAL-c expression vector generates fusion proteins which contain the MBP at the amino-terminal end of the protein. A construct, pMBot, in which the *C. botulinum* C fragment gene was expressed as a fusion with only the MBP was constructed (Figure 25). Fusion protein expression was induced from *E. coli* strains harboring the above plasmids, and induced protein was affinity purified on an amylose resin column.

i) Construction Of pBlueBot

In order to facilitate the cloning of the *C. botulinum* C fragment gene sequences into a number of desired constructs, the botulinal gene sequences were removed from pAlterBot and were inserted into the pBluescript plasmid (Stratagene) to generate pBlueBot (Figure 25). pBlueBot was constructed as follows. Bacteria containing the pAlterBot plasmid were grown in medium containing tetracycline and plasmid DNA was isolated using the QIAprep-spin Plasmid Kit (Qiagen). One microgram of pAlterBot DNA was digested with *Nco*I and the resulting 3' recessed sticky end was made blunt using the Klenow fragment of DNA polymerase I (here after the Klenow fragment). The pAlterBot DNA was then digested with *Hind*III to

release the botulinal gene sequences (the Bot insert) as a blunt (filled *NcoI* site)-
HindIII fragment. pBluescript vector DNA was prepared by digesting 200 ng of
pBluescript DNA with *SmaI* and *HindIII*. The digestion products from both plasmids
were resolved on an agarose gel. The appropriate fragments were removed from the
5 gel, mixed and purified utilizing the Prep-a-Gene kit (BioRad). The eluted DNA was
then ligated using T4 DNA ligase and used to transform competent DH5 α cells
(Gibco-BRL). Host cells were made competent for transformation using the calcium
chloride protocol of Sambrook *et al.*, *supra* at 1.82-1.83. Recombinant clones were
isolated and confirmed by restriction digestion using standard recombinant molecular
10 biology techniques (Sambrook *et al.*, *supra*). The resultant clone, pBlueBot, contains
several useful unique restriction sites flanking the Bot insert (*i.e.*, the *C. botulinum* C
fragment sequences derived from pAlterBot) as shown in Figure 25.

ii) Construction Of *C. difficile* /

C. botulinum / MBP Fusion Proteins

15 Constructs encoding fusions between the *C. difficile* toxin A gene and the
C. botulinum C fragment gene and the MBP were made utilizing the same recombinant
DNA methodology outlined above; these fusion proteins contained varying amounts of
the *C. difficile* toxin A repeat domain.

The pMABot clone contains a 2.4 kb insert derived from the *C. difficile* toxin
20 A gene fused to the Bot insert (*i.e.*, the *C. botulinum* C fragment sequences derived
from pAlterBot). pMABot (Figure 25) was constructed by mixing gel-purified DNA
from *NotI/HindIII* digested pBlueBot (the 1.2 kb Bot fragment), *SpeI/NotI* digested
pPA1100-2680 (the 2.4 kb *C. difficile* toxin A repeat fragment) and *XbaI/HindIII*
digested pMAL-c vector. Recombinant clones were isolated, confirmed by restriction
25 digestion and purified using the QIAprep-spin Plasmid Kit (Qiagen). This clone
expresses the toxin A repeats and the botulinal C fragment protein sequences as an in-
frame fusion with the MBP.

The pMCABot construct contains a 1.0 kb insert derived from the *C. difficile* toxin A gene fused to the Bot insert (*i.e.*, the *C. botulinum* C fragment sequences derived from pAlterBot). pMCABot was constructed by digesting the pMABot clone with *EcoRI* to remove the 5' end of the *C. difficile* toxin A repeat (see Figure 25, the pMAL-c vector contains a *EcoRI* site 5' to the *C. difficile* insert in the pMABot clone). The restriction sites were filled and religated together after gel purification. The resultant clone (pMCABot, Figure 25) generated an in-frame fusion between the MBP and the remaining 3' portion of the *C. difficile* toxin A repeat domain fused to the Bot gene.

The pMNABot clone contains the 1 kb *SpeI/EcoRI* (filled) fragment from the *C. difficile* toxin A repeat domain (derived from clone pPA1100-2680) and the 1.2 kb *C. botulinum* C fragment gene as a *NcoI* (filled)/*HindIII* fragment (derived from pAlterBot). These two fragments were inserted into the pMAL-c vector digested with *XbaI/HindIII*. The two insert fragments were generated by digestion of the appropriate plasmid with *EcoRI* (pPA1100-2680) or *NcoI* (pAlterBot) followed by treatment with the Klenow fragment. After treatment with the Klenow fragment, the plasmids were digested with the second enzyme (either *SpeI* or *HindIII*). All three fragments were gel purified, mixed and Prep-a-Gene purified prior to ligation. Following ligation and transformation, putative recombinants were analyzed by restriction analysis; the *EcoRI* site was found to be regenerated at the fusion junction, as was predicted for a fusion between the filled *EcoRI* and *NcoI* sites.

A construct encoding a fusion protein between the botulinal C fragment gene and the MBP gene was constructed (*i.e.*, this fusion lacks any *C. difficile* toxin A gene sequences) and termed pMBot. The pMBot construct was made by removal of the *C. difficile* toxin A sequences from the pMABot construct and fusing the C fragment gene sequences to the MBP. This was accomplished by digestion of pMABot DNA with *StuI* (located in the pMALc polylinker 5' to the *XbaI* site) and *XbaI* (located 3' to the *NotI* site at the toxA-Bot fusion junction), filling in the *XbaI* site using the Klenow fragment, gel purifying the desired restriction fragment, and ligating the blunt ends to

circularize the plasmid. Following ligation and transformation, putative recombinants were analyzed by restriction mapping of the Bot insert (*i.e.*, the *C. botulinum* C fragment sequences).

b) Expression Of *C. botulinum* C Fragment Fusion

Proteins In *E. coli*

Large scale (1 liter) cultures of the pMAL-c vector, and each recombinant construct described above in (a) were grown, induced, and soluble protein fractions were isolated as described in Example 18. The soluble protein extracts were chromatographed on amylose affinity columns to isolate recombinant fusion protein. The purified recombinant fusion proteins were analyzed by running samples on SDS-PAGE gels followed by Coomassie staining and by Western blot analysis as described [Williams *et al.*, (1994) *supra*]. In brief, extracts were prepared and chromatographed in column buffer (10 mM NaPO₄, 0.5 M NaCl, 10 mM β-mercaptoethanol, pH 7.2) over an amylose resin (New England Biolabs) column, and eluted with column buffer containing 10 mM maltose as described [Williams, *et al.* (1994), *supra*]. An SDS-PAGE gel containing the purified protein samples stained with Coomassie blue is shown in Figure 26.

In Figure 26, the following samples were loaded. Lanes 1-6 contain protein purified from *E. coli* containing the pMAL-c, pPA1870-2680, pMABot, pMNABot, pMCABot and pMBot plasmids, respectively. Lane 7 contains broad range molecular weight protein markers (BioRad).

The protein samples were prepared for electrophoresis by mixing 5 μl of eluted protein with 5 μl of 2X SDS-PAGE sample buffer (0.125 mM Tris-HCl, pH 6.8, 2 mM EDTA, 6% SDS, 20% glycerol, 0.025% bromophenol blue; β-mercaptoethanol is added to 5% before use). The samples were heated to 95°C for 5 min, then cooled and loaded on a 7.5% agarose SDS-PAGE gel. Broad range molecular weight protein markers were also loaded to allow estimation of the MW of identified fusion proteins.

After electrophoresis, protein was detected generally by staining the gel with Coomassie blue.

In all cases the yields were in excess of 20 mg fusion protein per liter culture (see Table 36) and, with the exception of the pMCABot protein, a high percentage (*i.e.*, greater than 20-50% of total eluted protein) of the eluted fusion protein was of a MW predicted for the full length fusion protein (Figure 26). It was estimated (by visual inspection) that less than 10% of the pMCABot fusion protein was expressed as the full length fusion protein.

TABLE 36

Yield Of Affinity Purified *C. botulinum* C Fragment / MBP Fusion Proteins

Construct	Yield (mg/liter of Culture)	Percentage Of Total Soluble Protein
pMABot	24	5.0
pMCABot	34	5.0
pMNABot	40	5.5
pMBot	22	5.0
pMA1870-2680	40	4.8

These results demonstrate that high level expression of intact *C. botulinum* C fragment/*C. difficile* toxin A fusion proteins in *E. coli* is feasible using the pMAL-c expression system. These results are in contrast to those reported by H. F. LaPenotiere, *et al.* (1993), *supra*. In addition, these results show that it is not necessary to fuse the botulinal C fragment gene to the *C. difficile* toxin A gene in order to produce a soluble fusion protein using the pMAL-c system in *E. coli*.

In order to determine whether the above-described botulinal fusion proteins were recognized by anti-*C. botulinum* toxin A antibodies, Western blots were performed. Samples containing affinity-purified proteins from *E. coli* containing the pMABot, pMCABot, pMNABot, pMBot, pMA1870-2680 or pMALc plasmids were analyzed. SDS-PAGE gels (7.5% acrylamide) were loaded with protein samples purified from each expression construct. After electrophoresis, the gels were blotted

and protein transfer was confirmed by Ponceau S staining (as described in Example 12b).

Following protein transfer, the blots were blocked by incubation for 1 hr at 20°C in blocking buffer [PBST (PBS containing 0.1% Tween 20 and 5% dry milk)].

5 The blots were then incubated in 10 ml of a solution containing the primary antibody; this solution comprised a 1/500 dilution of an anti-*C. botulinum* toxin A IgY PEG prep (described in Example 3) in blocking buffer. The blots were incubated for 1 hr at room temperature in the presence of the primary antibody. The blots were washed and developed using a rabbit anti-chicken alkaline phosphatase conjugate (Boehringer

10 Mannheim) as the secondary antibody as follows. The rabbit anti-chicken antibody was diluted to 1 µg/ml in blocking buffer (10 ml final volume per blot) and the blots were incubated at room temperature for 1 hour in the presence of the secondary antibody. The blots were then washed successively with PBST, BBS-Tween and 50 mM Na₂CO₃, pH 9.5. The blots were then developed in freshly-prepared alkaline phosphatase

15 substrate buffer (100 µg/ml nitro blue tetrazolium, 50 µg/ml 5-bromo-chloro-indolylphosphate, 5 mM MgCl₂ in 50 mM Na₂CO₃, pH 9.5). Development was stopped by flooding the blots with distilled water and the blots were air dried.

This Western blot analysis detected anti-*C. botulinum* toxin reactive proteins in the pMABot, pMCABot, pMNABot and pMBot protein samples (corresponding to the

20 predicted full length proteins identified above by Coomassie staining in Figure 26), but not in the pMA1100-2680 or pMALc protein samples.

These results demonstrate that the relevant fusion proteins purified on an amylose resin as described above in section a) contained immunoreactive *C botulinum* C fragment protein as predicted.

EXAMPLE 23

Generation Of Neutralizing Antibodies By Nasal Administration Of pMBot Protein

The ability of the recombinant botulinal toxin proteins produced in Example 22 to stimulate a systemic immune response against botulinal toxin epitopes was assessed. This example involved: a) the evaluation of the induction of serum IgG titers produced by nasal or oral administration of botulinal toxin-containing *C. difficile* toxin A fusion proteins and b) the *in vivo* neutralization of *C. botulinum* type A neurotoxin by anti- recombinant *C. botulinum* C fragment antibodies.

a) Evaluation Of The Induction Of Serum IgG Titers Produced By Nasal Or Oral Administration Of Botulinal Toxin-Containing *C. difficile* Toxin A Fusion Proteins

Six groups containing five 6 week old CF female rats (Charles River) per group were immunized nasally or orally with one of the following three combinations using protein prepared in Example 22: (1) 250 µg pMBot protein per rat (nasal and oral); 2) 250 µg pMABot protein per rat (nasal and oral); 3) 125 µg pMBot admixed with 125 µg pMA1870-2680 per rat (nasal and oral). A second set of 5 groups containing 3 CF female rats/group were immunized nasally or orally with one of the following combinations (4) 250 µg pMNABot protein per rat (nasal and oral) or 5) 250 µg pMAL-c protein per rat (nasal and oral).

The fusion proteins were prepared for immunization as follows. The proteins (in column buffer containing 10 mM maltose) were diluted in 0.1 M carbonate buffer, pH 9.5 and administered orally or nasally in a 200 µl volume. The rats were lightly sedated with ether prior to administration. The oral dosing was accomplished using a 20 gauge feeding needle. The nasal dosing was performed using a P-200 micropipettor (Gilson). The rats were boosted 14 days after the primary immunization using the techniques described above and were bled 7 days later. Rats from each group were

lightly etherized and bled from the tail. The blood was allowed to clot at 37°C for 1 hr and the serum was collected.

The serum from individual rats was analyzed using an ELISA to determine the anti-*C. botulinum* type A toxin IgG serum titer. The ELISA protocol used is a modification of that described in Example 13c. Briefly, 96-well microtiter plates (Falcon, Pro-Bind Assay Plates) were coated with *C. botulinum* type A toxoid (prepared as described in Example 3a) by placing 100 µl volumes of *C. botulinum* type A toxoid at 2.5 µg/ml in PBS containing 0.005% thimerosal in each well and incubating overnight at 4°C. The next morning, the coating suspensions were decanted and all wells were washed three times using PBS.

In order to block non-specific binding sites, 100 µl of blocking solution [0.5% BSA in PBS] was then added to each well and the plates were incubated for 1 hr at 37°C. The blocking solution was decanted and duplicate samples of 150 µl of diluted rat serum added to the first well of a dilution series. The initial testing serum dilution was 1:30 in blocking solution containing 0.5% Tween 20 followed by 5-fold dilutions into this solution. This was accomplished by serially transferring 30 µl aliquots to 120 µl blocking solution containing 0.5% Tween 20, mixing, and repeating the dilution into a fresh well. After the final dilution, 30 µl was removed from the well such that all wells contained 120 µl final volume. A total of 3 such dilutions were performed (4 wells total). The plates were incubated 1 hr at 37°C. Following this incubation, the serially diluted samples were decanted and the wells were washed six times using PBS containing 0.5% Tween 20 (PBST). To each well, 100 µl of a rabbit anti-Rat IgG alkaline phosphatase (Sigma) diluted (1/1000) in blocking buffer containing 0.5% Tween 20 was added and the plate was incubated for 1 hr at 37°C. The conjugate solutions were decanted and the plates were washed as described above, substituting 50 mM Na₂CO₃, pH 9.5 for the PBST in the final wash. The plates were developed by the addition of 100 µl of a solution containing 1 mg/ml para-nitro phenyl phosphate (Sigma) dissolved in 50 mM Na₂CO₃, 10 mM MgCl₂, pH 9.5 to each well, and incubating the plates at room temperature in the dark for 5-45 min. The absorbency of each well was measured at 410 nm using a Dynatech MR 700 plate reader. The

results are summarized in Tables 37 and 38 and represent mean serum reactivities of individual mice.

TABLE 37

Determination Of Anti-*C. botulinum* Type A Toxin Serum IgG Titers
Following Immunization With *C. botulinum* C Fragment-Containing Fusion Proteins

Route of Immunization		Nasal			Oral		
Immunogen	PRE- IMMUNE	pMBot	pMBot & pMA1870- 2680	pMABot	pMBot	pMBot & pMA1870- 2680	pMABot
Dilution							
1:30	0.080	1.040	1.030	0.060	0.190	0.080	0.120
1:150	0.017	0.580	0.540	0.022	0.070	0.020	0.027
1:750	0.009	0.280	0.260	0.010	0.020	0.010	0.014
1:3750	0.007	0.084	0.090	0.009	0.009	0.010	0.007
# Rats Tested		5	5	5	5	2	2

* Numbers represent the average values obtained from two ELISA plates, standardized utilizing the preimmune control.

TABLE 38

Determination Of Anti-*C. botulinum* Type A Toxin Serum IgG Titers
Following Immunization With *C. botulinum* C Fragment-Containing Fusion Proteins

Route of Immunization		Nasal		Oral	
Immunogen	PRE- IMMUNE	pMBot	pMABot	pMNABot	pMNABot
Dilution					
1:30	0.040	0.557	0.010	0.015	0.010
1:150	0.009	0.383	0.001	0.003	0.002
1:750	0.001	0.140	0.000	0.000	0.000
1:3750	0.000	0.040	0.000	0.000	0.000
# Rats Tested		1	1	3	3

The above ELISA results demonstrate that reactivity against the botulinal fusion proteins was strongest when the route of administration was nasal; only weak responses were stimulated when the botulinal fusion proteins were given orally.

Nasally delivered pMbot and pMBot admixed with pMA1870-2680 invoked the greatest serum IgG response. These results show that only the pMBot protein is necessary to induce this response, since the addition of the pMA1870-2680 protein did not enhance antibody response (Table 37). Placement of the *C. difficile* toxin A fragment between the MBP and the *C. botulinum* C fragment protein dramatically reduced anti-bot IgG titer (see results using pMABot, pMCABot and pMNABot proteins).

This study demonstrates that the pMBot protein induces a strong serum IgG response directed against *C. botulinum* type A toxin when nasally administered.

10 **b) *In Vivo* Neutralization Of *C. botulinum* Type A
 Neurotoxin By Anti- Recombinant *C. botulinum* C
 Fragment Antibodies**

15 The ability of the anti-*C. botulinum* type A toxin antibodies generated by nasal administration of recombinant botulinal fusion proteins in rats (Example 22) to neutralize *C. botulinum* type A toxin was tested in a mouse neutralization model. The mouse model is the art accepted method for detection of botulinal toxins in body fluids and for the evaluation of anti-botulinal antibodies [E.J. Schantz and D.A. Kautter, J. Assoc. Off. Anal. Chem. 61:96 (1990) and Investigational New Drug (BB-IND-3703) application by the Surgeon General of the Department of the Army to the Federal Food and Drug Administration]. The anti-*C. botulinum* type A toxin antibodies were prepared as follows.

20 Rats from the group given pMBot protein by nasal administration were boosted a second time with 250 µg pMBot protein per rat and serum was collected 7 days later. Serum from one rat from this group and from a preimmune rat was tested for anti-*C. botulinum* type A toxin neutralizing activity in the mouse neutralization model described below.

25 The LD₅₀ of a solution of purified *C. botulinum* type A toxin complex, obtained from Dr. Eric Johnson (University of Wisconsin Madison), was determined using the

intraperitoneal (IP) method of Schantz and Kautter [J. Assoc. Off. Anal. Chem. 61:96 (1978)] using 18-22 gram female ICR mice and was found to be 3500 LD₅₀/ml. The determination of the LD₅₀ was performed as follows. A Type A toxin standard was prepared by dissolving purified type A toxin complex in 25 mM sodium phosphate buffer, pH 6.8 to yield a stock toxin solution of 3.15 x 10⁷ LD₅₀/mg. The OD₂₇₈ of the solution was determined and the concentration was adjusted to 10-20 µg/ml. The toxin solution was then diluted 1:100 in gel-phosphate (30 mM phosphate, pH 6.4; 0.2% gelatin). Further dilutions of the toxin solution were made as shown below in Table 39. Two mice were injected IP with 0.5 ml of each dilution shown and the mice were observed for symptoms of botulism for a period of 72 hours.

TABLE 39

Determination Of The LD₅₀ Of Purified *C. botulinum* Type A Toxin Complex

Dilution	Number Dead At 72 hr
1:320	2/2
1:640	2/2
1:1280	2/2
1:2560	0/2 (sick after 72 hr)
1:5120	0/2 (no symptoms)

From the results shown in Table 39, the toxin titer was assumed to be between 2560 LD₅₀/ml and 5120 LD₅₀/ml (or about 3840 LD₅₀/ml). This value was rounded to 3500 LD₅₀/ml for the sake of calculation.

The amount of neutralizing antibodies present in the serum of rats immunized nasally with pMBot protein was then determined. Serum from two rats boosted with pMBot protein as described above and preimmune serum from one rat was tested as follows. The toxin standard was diluted 1:100 in gel-phosphate to a final concentration of 350 LD₅₀/ml. One milliliter of the diluted toxin standard was mixed with 25 µl of serum from each of the three rats and 0.2 ml of gel-phosphate. The mixtures were incubated at room temperature for 30 min with occasional mixing. Each of two mice were injected with IP with 0.5 ml of the mixtures. The mice were

observed for signs of botulism for 72 hr. Mice receiving serum from rats immunized with pMBot protein neutralized this challenge dose. Mice receiving preimmune rat serum died in less than 24 hr.

5 The amount of neutralizing anti-toxin antibodies present in the serum of rats immunized with pMBot protein was then quantitated. Serum antibody titrations were performed by mixing 0.1 ml of each of the antibody dilutions (see Table 40) with 0.1 ml of a 1:10 dilution of stock toxin solution (3.5×10^4 LD₅₀/ml) with 1.0 ml of gel-phosphate and injecting 0.5 ml IP into 2 mice per dilution. The mice were then observed for signs of botulism for 3 days (72 hr). The results are tabulated in
10 Table 39.

As shown in Table 40 pMBot serum neutralized *C. botulinum* type A toxin complex when used at a dilution of 1:320 or less. A mean neutralizing value of 168 IU/ml was obtained for the pMBot serum (an IU is defined as 10,000 mouse LD₅₀). This value translates to a circulating serum titer of about 3.7 IU/mg of serum protein.
15 This neutralizing titer is comparable to the commercially available bottled concentrated (Connaught Laboratories, Ltd.) horse anti-*C. botulinum* antiserum. A 10 ml vial of Connaught antiserum contains about 200 mg/ml of protein; each ml can neutralize 750 IU of *C. botulinum* type A toxin. After administration of one vial to a human, the circulating serum titer of the Connaught preparation would be approximately 25 IU/ml
20 assuming an average serum volume of 3 liters). Thus, the circulating anti-*C. botulinum* titer seen in rats nasally immunized with pMBot protein (168 IU/ml) is 6.7 time higher than the necessary circulation titer of anti-*C. botulinum* antibody needed to be protective in humans.

TABLE 40

Quantitation Of Neutralizing Antibodies In pMBot Sera

Dilution	pMBot ^a	
	Rat 1	Rat 2
1:20	2/2	2/2
1:40	2/2	2/2
1:80	2/2	2/2
1:160	2/2	2/2
1:320	2/2 ^b	2/2 ^b
1:640	0/2	0/2
1:1280	0/2	0/2
1:2560	0/2	0/2

^a Numbers represent the number of mice surviving at 72 hours which received serum taken from rats immunized with the pMBot protein.

^b These mice survived but were sick after 72 hr.

These results demonstrate that antibodies capable of neutralizing *C. botulinum* type A toxin are induced when recombinant *C. botulinum* C fragment fusion protein produced in *E. coli* is used as an immunogen.

EXAMPLE 24

Production Of Soluble *C. botulinum* C Fragment

Protein Substantially Free Of Endotoxin Contamination

Example 23 demonstrated that neutralizing antibodies are generated by immunization with the pMBot protein expressed in *E. coli*. These results showed that the pMBot fusion protein is a good vaccine candidate. However, immunogens suitable for use as vaccines should be pyrogen-free in addition to having the capability of inducing neutralizing antibodies. Expression clones and conditions that facilitate the production of *C. botulinum* C fragment protein for utilization as a vaccine were developed.

The example involved: (a) determination of pyrogen content of the pMBot protein; (b) generation of *C. botulinum* C fragment protein free of the MBP; (c) expression of *C. botulinum* C fragment protein using various expression vectors; and (d) purification of soluble *C. botulinum* C fragment protein substantially free of significant endotoxin contamination.

a) Determination Of The Pyrogen Content Of The pMBot Protein

In order to use a recombinant antigen as a vaccine in humans or other animals, the antigen preparation must be shown to be free of pyrogens. The most significant pyrogen present in preparations of recombinant proteins produced in gram-negative bacteria, such as *E. coli*, is endotoxin [F.C. Pearson, *Pyrogens: endotoxins, LAL testing and depyrogenation*, (1985) Marcel Dekker, New York, pp. 23-56]. To evaluate the utility of the pMBot protein as a vaccine candidate, the endotoxin content in MBP fusion proteins was determined.

The endotoxin content of recombinant protein samples was assayed utilizing the Limulus assay (LAL kit; Associates of Cape Cod) according to the manufacturer's instructions. Samples of affinity-purified pMal-c protein and pMA1870-2680 were found to contain high levels of endotoxin [$>50,000$ EU/mg protein; EU (endotoxin unit)]. This suggested that MBP- or toxin A repeat-containing fusions with the botulinal C fragment should also contain high levels of endotoxin. Accordingly, removal of endotoxin from affinity-purified pMal-c and pMBot protein preparations was attempted as follows.

Samples of pMal-c and pMBot protein were depyrogenated with polymyxin to determine if the endotoxin could be easily removed. The following amount of protein was treated: 29 ml at $4.8 \text{ OD}_{280}/\text{ml}$ for pMal-c and 19 mls at $1.44 \text{ OD}_{280}/\text{ml}$ for pMBot. The protein samples were dialyzed extensively against PBS and mixed in a 50 ml tube (Falcon) with 0.5 ml PBS-equilibrated polymyxin B (Affi-Prep Polymyxin, BioRad). The samples were allowed to mix by rotating the tubes overnight at 4°C .

The polymyxin was pelleted by centrifugation for 30 min in a bench top centrifuge at maximum speed (approximately 2000 x g) and the supernatant was removed. The recovered protein (in the supernatant) was quantified by OD₂₈₀, and the endotoxin activity was assayed by LAL. In both cases only approximately 1/3 of the input protein was recovered and the polymyxin-treated protein retained significant endotoxin contamination (approximately 7000 EU/mg of pMBot).

The depyrogenation experiment was repeated using an independently purified pMal-c protein preparation and similar results were obtained. From these studies it was concluded that significant levels of endotoxin copurifies with these MBP fusion proteins using the amylose resin. Furthermore, this endotoxin cannot be easily removed by polymyxin treatment.

These results suggest that the presence of the MBP sequences on the fusion protein complicated the removal of endotoxin from preparations of the pMBot protein.

b) Generation Of *C. botulinum* C Fragment Protein Free Of The MBP

It was demonstrated that the pMBot fusion protein could not be easily purified from contaminating endotoxin in section a) above. The ability to produce a pyrogen-free (*e.g.*, endotoxin-free) preparation of soluble botulinal C fragment protein free of the MBP tag was next investigated. The pMBot expression construct was designed to facilitate purification of the botulinal C fragment from the MBP tag by cleavage of the fusion protein by utilizing an engineered Factor Xa cleavage site present between the MBP and the botulinal C fragment. The Factor Xa cleavage was performed as follows.

Factor Xa (New England Biolabs) was added to the pMBot protein (using a 0.1-1.0% Factor Xa/pMBot protein ratio) in a variety of buffer conditions [*e.g.*, PBS-NaCl (PBS containing 0.5 M NaCl), PBS-NaCl containing 0.2% Tween 20, PBS, PBS containing 0.2% Tween 20, PBS-C (PBS containing 2 mM CaCl₂), PBS-C containing either 0.1 or 0.5 % Tween 20, PBS-C containing either 0.1 or 0.5% NP-40, PBS-C containing either 0.1 or 0.5% Triton X-100, PBS-C containing 0.1% sodium

deoxycholate, PBS-C containing 0.1% SDS]. The Factor Xa digestions were incubated for 12-72 hrs at room temperature.

The extent of cleavage was assessed by Western blot or Coomassie blue staining of proteins following electrophoresis on denaturing SDS-PAGE gels, as described in Example 22. Cleavage reactions (and control samples of uncleaved pMBot protein) were centrifuged for 2 min in a microfuge to remove insoluble protein prior to loading the samples on the gel. The Factor Xa treated samples were compared with uncleaved, uncentrifuged pMBot samples on the same gel. The results of this analysis is summarized below.

1) Most (about 90%) pMBot protein could be removed by centrifugation, even when uncleaved control samples were utilized. This indicated that the pMBot fusion protein was not fully soluble (*i.e.*, it exists as a suspension rather than as a solution). [This result was consistent with the observation that most affinity-purified pMBot protein precipitates after long term storage (>2 weeks) at 4°C. Additionally, the majority (*i.e.*, 75%) of induced pMBot protein remains in the pellet after sonication and clarification of the induced *E. coli*. Resuspension of these insoluble pellets in PBS followed by sonication results in partial solubilization of the insoluble pMBot protein in the pellets.]

2) The portion of pMBot protein that is fully in solution (about 10% of pMBot protein) is completely cleaved by Factor Xa, but the cleaved (released) botulinal C fragment is relatively insoluble such that only the cleaved MBP remains fully in solution.

3) None of the above reaction conditions enhanced solubility without also reducing effective cleavage. Conditions that effectively solubilized the cleaved botulinal C fragment were not identified.

4) The use of 0.1% SDS in the buffer used for Factor Xa cleavage enhanced the solubility of the pMBot protein (all of pMBot protein was soluble). However, the presence of the SDS prevented any cleavage of the fusion protein with Factor Xa.

5) Analysis of pelleted protein from the cleavage reactions indicated that both full length pMBot (*i.e.*, uncleaved) and cleaved botulinal C fragment protein precipitated during incubation.

5 These results demonstrate that purification of soluble botulinal C fragment protein after cleavage of the pMBot fusion protein is complicated by the insolubility of both the pMBot protein and the cleaved botulinal C fragment protein.

c) Expression Of *C. botulinum* C Fragment Using Various Expression Vectors

10 In order to determine if the solubility of the botulinal C fragment was enhanced by expressing the C fragment protein as a native protein, an N-terminal His-tagged protein or as a fusion with glutathione-S-transferase (GST), alternative expression plasmids were constructed. These expression constructs were generated utilizing the methodologies described in Example 22. Figure 27 provides a schematic representation of the vectors described below.

15 In Figure 27, the following abbreviations are used. pP refers to the pET23 vector. pHIS refers to the pETHisa vector. pBlue refers to the pBluescript vector. pM refers to the pMAL-c vector and pG refers to the pGEX3T vector (described in Example 11). The solid black lines represent *C. botulinum* C fragment gene sequences; the solid black ovals represent the MBP; the hatched ovals represent GST; "HHHHH" represents the poly-histidine tag. In Figure 27, when the name for a restriction enzyme appears inside parenthesis, this indicates that the restriction site was destroyed during construction. An asterisk appearing with the name for a restriction enzyme indicates that this restriction site was recreated at a cloning junction.

20

i) Construction Of pPBot

25 In order to express the *C. botulinum* C fragment as a native (*i.e.*, non-fused) protein, the pPBot plasmid (shown schematically in Figure 27) was constructed as follows. The C fragment sequences present in pAlterBot (Example 22) were removed by digestion of pAlterBot with *Nco*I and *Hind*III. The *Nco*I/*Hind*III C fragment insert

was ligated to pETHisa vector (described in Example 18b) which was digested with *NcoI* and *HindIII*. This ligation creates an expression construct in which the *NcoI*-encoded methionine of the botulinal C fragment is the initiator codon and directs expression of the native botulinal C fragment. The ligation products were used to transform competent BL21(DE3)pLysS cells (Novagen). Recombinant clones were identified by restriction mapping.

ii) Construction Of pHisBot

In order to express the *C. botulinum* C fragment containing a poly-histidine tag at the amino-terminus of the recombinant protein, the pHisBot plasmid (shown schematically in Figure 27) was constructed as follows. The *NcoI/HindIII* botulinal C fragment insert from pAlterbot was ligated into the pETHisa vector which was digested with *NheI* and *HindIII*. The *NcoI* (on the C fragment insert) and *NheI* (on the pETHisa vector) sites were filled in using the Klenow fragment prior to ligation; these sites were then blunt end ligated (the *NdeI* site was regenerated at the clone junction as predicted). The ligation products were used to transform competent BL21(DE3)pLysS cells and recombinant clones were identified by restriction mapping.

The resulting pHisBot clone expresses the botulinal C fragment protein with a histidine-tagged N-terminal extension having the following sequence: MetGlyHisHisHisHisHisHisHisHisHisSerSerGlyHisIleGluGlyArgHisMetAla (SEQ ID NO:24); the amino acids encoded by the botulinal C fragment gene are underlined and the vector encoded amino acids are presented in plain type. The nucleotide sequence present in the pETHisa vector which encodes the pHisBot fusion protein is listed in SEQ ID NO:25. The amino acid sequence of the pHisBot protein is listed in SEQ ID NO:26.

iii) Construction Of pGBot

The botulinal C fragment protein was expressed as a fusion with the glutathione-S-transferase protein by constructing the pGBot plasmid (shown schematically in Figure 27). This expression construct was created by cloning the
5 *NotI/SaI* C fragment insert present in pBlueBot (Example 22) into the pGEX3T vector which was digested with *SmaI* and *XhoI*. The *NotI* site (present on the botulinal fragment) was made blunt prior to ligation using the Klenow fragment. The ligation products were used to transform competent BL21 cells.

Each of the above expression constructs were tested by restriction digestion to
10 confirm the integrity of the constructs.

Large scale (1 liter) cultures of pPBot [BL21(DE3)pLysS host], pHisBot [BL21(DE3)pLysS host] and pGBot (BL21 host) were grown in 2X YT medium and induced (using IPTG to 0.8-1.0 mM) for 3 hrs as described in Example 22. Total, soluble and insoluble protein preparations were prepared from 1 ml aliquots of each
15 large scale culture [Williams *et al.* (1994), *supra*] and analyzed by SDS-PAGE. No obvious induced band was detectable in the pPBot or pHisBot samples by Coomassie staining, while a prominent insoluble band of the anticipated MW was detected in the pGBot sample. Soluble lysates of the pGBot large scale (resuspended in PBS) or pHisBot large scale [resuspended in Novagen 1X binding buffer (5 mM imidazole, 0.5
20 M NaCl, 20 mM Tris-HCl, pH 7.9)] cultures were prepared and used to affinity purify soluble affinity-tagged protein as follows.

The pGBot lysate was affinity purified on a glutathione-agarose resin (Pharmacia) exactly as described in Smith and Corcoran [Current Protocols in Molecular Biology, Supplement 28 (1994), pp. 16.7.1-16.7.7]. The pHisBot protein
25 was purified on the His-Bind resin (Novagen) utilizing the His-bind buffer kit (Novagen) exactly as described by manufacturer.

Samples from the purification of both the pGBot and pHisBot proteins (including uninduced, induced, total, soluble, and affinity-purified eluted protein) were resolved on SDS-PAGE gels. Following electrophoresis, proteins were analyzed by

Coomassie staining or by Western blot detection utilizing a chicken anti-*C. botulinum* Type A toxoid antibody (as described in Example 22).

These studies showed that the pGBot protein was almost entirely insoluble under the utilized conditions, while the pHisBot protein was soluble. Affinity purification of the pHisBot protein on this first attempt was inefficient, both in terms of yield (most of the immunoreactive botulinal protein did not bind to the His-bind resin) and purity (the botulinal protein was estimated to comprise approximately 20% of the total eluted protein).

**d) Purification Of Soluble *C. botulinum* C Fragment
Protein Substantially Free Of Endotoxin
Contamination**

The above studies showed that the pHisBot protein was expressed in *E. coli* as a soluble protein. However, the affinity purification of this protein on the His-bind resin was very inefficient. In order to improve the affinity purification of the soluble pHisBot protein (in terms of both yield and purity), an alternative poly-histidine binding affinity resin (Ni-NTA resin; Qiagen) was utilized. The Ni-NTA resin was reported to have a superior binding affinity ($K_d = 1 \times 10^{-13}$ at pH 8.0; Qiagen user manual) relative to the His-bind resin.

A soluble lysate (in Novagen 1X binding buffer) from an induced 1 liter 2X YT culture was prepared as described above. Briefly, the culture of pHisBot [Bl21(DE3)pLysS host] was grown at 37°C to an OD_{600} of 0.7 in 1 liter of 2X YT medium containing 100 µg/ml ampicillin, 34 µg/ml chloramphenicol and 0.2% glucose. Protein expression was induced by the addition of IPTG to 1 mM. Three hours after the addition of the IPTG, the cells were cooled for 15 min in a ice water bath and then centrifuged 10 min at 5000 rpm in a JA10 rotor (Beckman) at 4°C. The pellets were resuspended in a total volume of 40 mls Novagen 1X binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), transferred to two 35 ml Oakridge tubes and frozen at -70°C for at least 1 hr. The tubes were thawed and the cells were lysed by sonication (4 X 20 second bursts using a Branson Sonifier 450

with a power setting of 6-7) on ice. The suspension was clarified by centrifugation for 20 min at 9,000 rpm (10,000 x g) in a JA-17 rotor (Beckman).

The soluble lysate was brought to 0.1% NP40 and then was batch absorbed to 7 ml of a 1:1 slurry of Ni-NTA resin:binding buffer by stirring for 1 hr at 4°C. The slurry was poured into a column having an internal diameter of 1 or 2.5 cm (BioRad). The column was then washed sequentially with 15 mls of Novagen 1X binding buffer containing 0.1% NP40, 15 ml of Novagen 1X binding buffer, 15 ml wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and 15 ml NaHPO₄ wash buffer (50 mM NaHPO₄, pH 7.0, 0.3 M NaCl, 10 % glycerol). The bound protein was eluted by protonation of the resin using elution buffer (50 mM NaHPO₄, pH 4.0, 0.3 M NaCl, 10 % glycerol). The eluted protein was stored at 4°C.

Samples of total, soluble and eluted protein were resolved by SDS-PAGE. Protein samples were prepared for electrophoresis as described in Example 22b. Duplicate gels were stained with Coomassie blue to visualize the resolved proteins and *C. botulinum* type A toxin-reactive protein was detected by Western blot analysis as described in Example 22b. A representative Coomassie stained gel is shown in Figure 28. In Figure 28, the following samples were loaded on the 12.5% acrylamide gel. Lanes 1-4 contain respectively total protein, soluble protein, soluble protein present in the flow-through of the Ni-NTA column and affinity-purified pHisBot protein (*i.e.*, protein released from the Ni-NTA resin by protonation). Lane 5 contains high molecular weight protein markers (BioRad).

The purification of pHisBot protein resulted in a yield of 7 mg of affinity purified protein from a 1 liter starting culture of BL21(DE3)pLysS cells harboring the pHisBot plasmid. The yield of purified pHisBot protein represented approximately 0.4% of the total soluble protein in the induced culture. Analysis of the purified pHisBot protein by SDS-PAGE revealed that at least 90-95% of the protein was present as a single band (Figure 28) of the predicted MW (50 kD). This 50 kD protein band was immunoreactive with anti-*C. botulinum* type A toxin antibodies. The extinction coefficient of the protein preparation was determined to be 1.4 (using the Pierce BCA assay) or 1.45 (using the Lowry assay) OD₂₈₀ per 1 mg/ml solution.

Samples of pH neutralized eluted pHisBot protein were resolved on a KB 803 HPLC column (Shodex). Although His-tagged proteins are retained by this sizing column (perhaps due to the inherent metal binding ability of the proteins), the relative mobility of the pHisBot protein was consistent with that expected for a non-aggregated protein in solution. Most of the induced pHisBot protein was determined to be soluble under the growth and solubilization conditions utilized above (*i.e.*, greater than 90% of the pHisBot protein was found to be soluble as judged by comparison of the levels of pHisBot protein seen in total and soluble protein samples prepared from BL21(DE3)pLysS cells containing the pHisBot plasmid). SDS-PAGE analysis of samples obtained after centrifugation, extended storage at -20°C, and at least 2 cycles of freezing and thawing detected no protein loss (due to precipitation), indicating that the pHisBot protein is soluble in the elution buffer (*i.e.*, 50 mM NaHPO₄, pH 4.0, 0.3 M NaCl, 10 % glycerol).

Determination of endotoxin contamination in the affinity purified pHisBot preparation (after pH neutralization) using the LAL assay (Associates of Cape Cod) detected no significant endotoxin contamination. The assay was performed using the endpoint chromogenic method (without diazo-coupling) according to the manufacturer's instructions. This method can detect concentrations of endotoxin greater than or equal to 0.03 EU/ml (EU refers to endotoxin units). The LAL assay was run using 0.5 ml of a solution comprising 0.5 mg pHisBot protein in 50 mM NaHPO₄, pH 7.0, 0.3 M NaCl, 10 % glycerol; 30-60 EU were detected in the 0.5 ml sample. Therefore, the affinity purified pHisBot preparation contains 60-120 EU/mg of protein. FDA Guidelines for the administration of parenteral drugs require that a composition to be administered to a human contain less than 5 EU/kg body weight (The average human body weight is 70 kg; therefore up to 349 EU units can be delivered in a parental dose.). Because very small amount of protein are administered in a vaccine preparation (generally in the range of 10-500 µg of protein), administration of affinity purified pHisBot containing 60-120 EU/mg protein would result in delivery of only a small percentage of the permissible endotoxin load. For example, administration of 10-500 µg of purified pHisBot to a 70 kg human, where

the protein preparation contains 60 EU/mg protein, results in the introduction of only 0.6 to 30 EU [*i.e.*, 0.2 to 8.6% of the maximum allowable endotoxin burden per parenteral dose (less than 5 EU/kg body weight)].

5 The above results demonstrate that endotoxin (LPS) does not copurify with the pHisBot protein using the above purification scheme. Preparations of recombinantly produced pHisBot protein containing lower levels of endotoxin (less than or equal to 2 EU/ mg recombinant protein) may be produced by washing the Ni-NTA column with wash buffer until the OD₂₈₀ returns to baseline levels (*i.e.*, until no more UV-absorbing material comes off of the column).

10 The above results illustrate a method for the production and purification of soluble, botulinal C fragment protein substantially free of endotoxin.

EXAMPLE 25

Optimization Of The Expression And Purification Of pHisBot Protein

15 The results shown in Example 24d demonstrated that the pHisBot protein is an excellent candidate for use as a vaccine as it could be produced as a soluble protein in *E. coli* and could be purified free of pyrogen activity. In order to optimize the expression and purification of the pHisBot protein, a variety of growth and purification conditions were tested.

a) Growth Parameters

20 i) Host Strains

25 The influence of the host strain utilized upon the production of soluble pHisBot protein was investigated. A large scale purification of pHisBot was performed [as described in Example 24d above] using the BL21(DE3) host (Novagen) rather than the BL21(DE3)pLysS host. The deletion of the pLysS plasmid in the BL21(DE3) host yielded higher levels of expression due to de-repression of the plasmid's T7-lac promoter. However, the yield of affinity-purified soluble recombinant protein was very low (approximately 600 µg/ liter culture) when purified under conditions identical

to those described in Example 24d above. This result was due to the fact that expression in the BL21(DE3) host yielded very high level expression of the pHisBot protein as insoluble inclusion bodies as shown by SDS-PAGE analysis of protein prepared from induced BL21(DE3) cultures (Figure 29, lanes 1-7, described below).

5 These results demonstrate that the pHisBot protein is not inherently toxic to *E. coli* cells and can be expressed to high levels using the appropriate promoter/host combination.

Figure 29 shows a Coomassie blue stained SDS-PAGE gel (12.5% acrylamide) onto which extracts prepared from BL21(DE3) cells containing the pHisBot plasmid were loaded. Each lane was loaded with 2.5 μ l protein sample mixed with 2.5 μ l of 10 2X SDS sample buffer. The samples were handled as described in Example 22b. The following samples were applied to the gel. Lanes 1-7 contain protein isolated from the BL21(DE3) host. Lanes 8-14 contain proteins isolated from the BL21(DE3)pLysS host. Total protein was loaded in lanes 1, 2, 4, 6, 8, 10 and 12. Soluble protein was 15 loaded in Lanes 3, 5, 7, 9, 11 and 13. Lane 1 contains protein from uninduced host cells. Lanes 2-13 contain protein from host cells induced for 3 hours. IPTG was added to a final concentration of 0.1 mM (Lanes 6-7), 0.3 mM (Lanes 4-5) or 1.0 mM (Lanes 2, 3, 8-13). The cultures were grown in LB broth (Lanes 8-9), 2X YT broth (Lanes 10-11) or terrific broth (Lanes 1-7, 12-13). The pHisBot protein seen in Lanes 20 3, 5 and 7 is insoluble protein which spilled over from Lanes 2, 4 and 6, respectively. High molecular weight protein markers (BioRad) were loaded in Lane 14.

A variety of expression conditions were tested to determine if the BL21(DE3) host could be utilized to express soluble pHisBot protein at suitably high levels (*i.e.*, about 10 mg/ml). The conditions altered were temperature (growth at 37 or 30°C), 25 culture medium (2X YT, LB or Terrific broth) and inducer levels (0.1, 0.3 or 1.0 mM IPTG). All combinations of these variables were tested and the induction levels and solubility was then assessed by SDS-PAGE analysis of total and soluble extracts [prepared from 1 ml samples as described in Williams *et al.*, (1994), *supra*].

All cultures were grown in 15 ml tubes (Falcon #2057). All culture medium 30 was prewarmed overnight at the appropriate temperature and were supplemented with

100 µg/ml ampicillin and 0.2% glucose. Terrific broth contains 12 g/l bacto-tryptone, 24 g/l bacto-yeast extract and 100 ml/l of a solution comprising 0.17 M KH_2PO_4 , 0.72 M K_2HPO_4 . Cultures were grown in a incubator on a rotating wheel (to ensure aeration) to an OD_{600} of approximately 0.4, and induced by the addition of IPTG. In all cases, high level expression of insoluble pHisBot protein was observed, regardless of temperature, medium or inducer concentration.

The effect of varying the concentration of IPTG upon 2X YT cultures grown at 23°C was then investigated. IPTG was added to a final concentration of either 1 mM, 0.1 mM, 0.05 mM or 0.01 mM. At this temperature, similar levels of pHis Bot protein was induced in the presence of either 1 or 0.1 mM IPTG; these levels of expression was lower than that observed at higher temperatures. Induced protein levels were reduced at 0.05 mM IPTG and absent at 0.01 mM IPTG (relative to 1.0 and 0.1 mM IPTG inductions at 23°C). However, no conditions were observed in which the induced pHisBot protein was soluble in this host. Thus, although expression levels are superior in the BL21(DE3) host (as compared to the BL21(DE3)pLysS host), conditions that facilitate the production of soluble protein in this host could not be identified.

These results demonstrate that production of soluble pHisBot protein was achieved using the BL21(DE3)pLysS host in conjunction with the T7-lac promoter.

ii) Effect Of Varying Temperature, Medium And IPTG Concentration And Length Of Induction

The effect growing the host cells in various mediums upon the expression of recombinant botulinal protein from the pHisBot expression construct [in the BL21(DE3)pLysS host] was investigated. BL21(DE3)pLysS cells containing the pHisBot plasmid were grown in either LB, 2X YT or Terrific broth at 37°C. The cells were induced using 1 mM IPTG for a 3 hr induction period. Expression of pHisBot protein was found to be the highest when the cells were grown in 2X YT broth (see Figure 29, lanes 8-13).

The cells were then grown at 30°C in 2X YT broth and the concentration of IPTG was varied from 1.0, 0.3 or 0.1 mM and the length of induction was either 3 or 5 hours. Expression of pHisBot protein was similar at all 3 inducer concentrations utilized and the levels of induced protein were higher after a 5 hr induction as compared to a 3 hr induction.

Using the conditions found to be optimal for the expression of pHisBot protein, a large scale culture was grown in order to provide sufficient material for a large scale purification of the pHisBot protein. Three 1 liter cultures were grown in 2X YT medium containing 100 µg/ml ampicillin, 34 µg/ml chloramphenicol and 0.2% glucose. The cultures were grown at 30°C and were induced with 1.0 mM IPTG for a 5 hr period. The cultures were harvested and a soluble lysate were prepared as described in Example 18. A large scale purification was performed as described in Example 24d with the exception that except the soluble lysate was batch absorbed for 3 hours rather than for 1 hour. The final yield was 13 mg pHisBot protein/liter culture. The pHisBot protein represented 0.75% of the total soluble protein.

The above results demonstrate growth conditions under which soluble pHisBot protein is produced (*i.e.*, use of the BL21(DE3)pLysS host, 2X YT medium, 30°C, 1.0 mM IPTG for 5 hours).

b) Optimization Of Purification Parameters

For optimization of purification conditions, large scale cultures (3 X 1 liter) were grown at 30°C and induced with 1 mM IPTG for 5 hours as described above. The cultures were pooled, distributed to centrifuge bottles, cooled and pelleted as described in Example 24d. The cell pellets were frozen at -70°C until used. Each cell pellet represented 1/3 of a liter starting culture and individual bottles were utilized for each optimization experiment described below. This standardized the input bacteria used for each experiment, such that the yields of affinity purified pHisBot protein could be compared between different optimization experiments.

i) Binding Specificity (pH Protonation)

A lysate of pHisBot culture was prepared in PBS (pH 8.0) and applied to a 3 ml Ni-NTA column equilibrated in PBS (pH 8.0) using a flow rate of 0.2 ml/min (3-4 column volumes/hr) using an Econo chromatography system (BioRad). The column was washed with PBS (pH 8.0) until the absorbance (OD_{280}) of the elute was at baseline levels. The flow rate was then increased to 2 ml/min and the column was equilibrated in PBS (pH 7.0). A pH gradient (pH 7.0 to 4.0 in PBS) was applied in order to elute the bound pHisBot protein from the column. Fractions were collected and aliquots were resolved on SDS-PAGE gels. The PAGE gels were subjected to Western blotting and the pHisBot protein was detected using a chicken anti-*C. botulinum* Type A toxoid antibody as described in Example 22.

From the Western blot analysis it was determined that the pHisBot protein begins to elute from the Ni-NTA column at pH 6.0. This is consistent with the predicted elution of a His-tagged protein monomer at pH 5.9.

These results demonstrate that the pH at which the pHisBot protein is protonated (released) from Ni-NTA resin in PBS buffer is pH 6.0.

ii) Binding Specificity (Imidazole Competition)

In order to define purification conditions under which the native *E. coli* proteins could be removed from the Ni-NTA column while leaving the pHisBot protein bound to the column, the following experiment was performed. A lysate of pHisBot culture was prepared in 50 mM NaHPO_4 , 0.5 M NaCl, 8 mM imidazole (pH 7.0). This lysate was applied to a 3 ml Ni-NTA column equilibrated in 50 mM NaHPO_4 , 0.5 M NaCl (pH 7.0) using an Econo chromatography system (BioRad). A flow rate of 0.2 ml/min (3-4 column volumes/hr) was utilized. The column was washed with 50 mM NaHPO_4 , 0.5 M NaCl (pH 7.0) until the absorbance of the elute returned to baseline. The flow rate was then increased to 2 ml/min.

The column was eluted using an imidazole step gradient [in 50 mM NaHPO_4 , 0.5 M NaCl (pH 7.0)]. Elution steps were 20 mM, 40 mM, 60 mM, 80 mM, 100 mM,

200 mM, 1.0 M imidazole, followed by a wash using 0.1 mM EDTA (to strip the nickel from the column and remove any remaining protein). In each step, the wash was continued until the OD₂₈₀ returned to baseline. Fractions were resolved on SDS-PAGE gels, Western blotted, and pHisBot protein detected using a chicken anti-*C. botulinum* Type A toxoid antibody as described in Example 22. Duplicate gels were stained with Coomassie blue to detect eluted protein in each fraction.

The results of the PAGE analysis showed that most of the non-specifically binding bacterial protein was removed by the 20 mM imidazole wash, with the remaining bacterial proteins being removed in the 40 and 60 mM imidazole washes. The pHisBot protein began to elute at 100 mM imidazole and was quantitatively eluted in 200 mM imidazole.

These results precisely defined the window of imidazole wash stringency that optimally removes *E. coli* proteins from the column while specifically retaining the pHisBot protein in this buffer. These results provided conditions under which the pHisBot protein can be purified free of contaminating host proteins.

iii) Purification Buffers And Optimized Purification Protocols

A variety of purification parameters were tested during the development of an optimized protocol for batch purification of soluble pHisBot protein. The results of these analyses are summarized below.

Batch purifications were performed (as described in Example 24d) using several buffers to determine if alternative buffers could be utilized for binding of the pHisBot protein to the Ni-NTA column. It was determined that quantitative binding of pHisBot protein to the Ni-NTA resin was achieved in either Tris-HCl (pH 7.9) or NaHPO₄ (pH 8.0) buffers. Binding of the pHisBot protein in NaHPO₄ buffer was not inhibited using 5 mM, 8 mM or 60 mM imidazole. Quantitative elution of bound pHisBot protein was obtained in buffers containing 50 mM NaHPO₄, 0.3 M NaCl (pH 3.5-4.0), with or without 10% glycerol. However, quantitation of soluble affinity purified pHisBot protein before and after a freeze thaw (following several weeks storage of the affinity

purified elute at -20°C) revealed that 94% of the protein was recovered using the glycerol-containing buffer, but only 68% of the protein was recovered when the buffer lacking glycerol was employed. This demonstrates that glycerol enhanced the solubility of the pHisBot protein in this low pH buffer when the eluted protein was stored at freezing temperatures (*e.g.*, -20°C). Neutralization of pH by addition of NaH₂PO₄ buffer did not result in obvious protein precipitation.

It was determined that quantitative binding of pHisBot protein using the batch format occurred after 3 hrs (Figure 30), but not after 1 hr of binding at 4°C (the resin was stirred during binding). Figure 30 depicts a Coomassie blue stained SDS-PAGE gel (7.5% acrylamide) containing samples of proteins isolated during the purification of pHisBot protein from lysate prepared from the BL21(DE3)pLysS host. Each lane was loaded with 5 µl of protein sample mixed with 5 µl of 2X sample buffer and processed as described in Example 22b. Lane 1 contains high molecular weight protein markers (BioRad). Lanes 2 and 3 contain protein eluted from the Ni-NTA resin. Lane 4 contains soluble protein after a 3 hr batch incubation with the Ni-NTA resin. Lanes 5 and 6 contain soluble and total protein, respectively. Figure 30 demonstrates that the pHisBot protein is completely soluble [compare Lanes 5 and 6 which show that a similar amount of the 50 kD pHisBot protein is seen in both; if a substantial amount (greater than 20%) of the pHisBot protein were partially insoluble in the host cell, more pHisBot protein would be seen in lane 6 (total protein) as compared to lane 5 (soluble protein)]. Figure 30 also demonstrates that the pHisBot protein is completely removed from the lysate after batch absorption with the Ni-NTA resin for 3 hours (compare Lanes 4 and 5).

The reported high affinity interaction of the Ni-NTA resin with His-tagged proteins ($K_d = 1 \times 10^{-13}$ at pH 8.0) suggested that it should be possible to manipulate the resin-protein complexes without significant release of the bound protein. Indeed, it was determined that after the recombinant protein was bound to the Ni-NTA resin, the resin-pHisBot protein complex was highly stable and remained bound following repeated rounds of centrifugation of the resin for 2 min at 1600 x g. When this centrifugation step was performed in a 50 ml tube (Falcon), a tight resin pellet formed.

This allowed the removal of spent soluble lysate by pouring off the supernatant followed by resuspension of the pellet in wash buffer. Further washes can be performed by centrifugation. The ability to perform additional washes permits the development of protocols for batch absorption of large volumes of lysate with removal of the lysate being performed simply by centrifugation following binding of the recombinant protein to the resin.

A simplified, integrated purification protocol was developed as follows. A soluble lysate was made by resuspending the induced cell pellet in binding buffer [50 mM NaHPO₄, 0.5 M NaCl, 60 mM imidazole (pH 8.0)], sonicating 4 x 20 sec and centrifuging for 20 min at 10,000 x g. NP-40 was added to 0.1% and Ni-NTA resin (equilibrated in binding buffer) was added. Eight milliliters of a 1:1 slurry (resin:binding buffer) was used per liter of starting culture. The mixture was stirred for 3 hrs at 4°C. The slurry was poured into a column having a 1 cm internal diameter (BioRad), washed with binding buffer containing 0.1% NP40, then binding buffer until baseline was established (these steps may alternatively be performed by centrifugation of the resin, resuspension in binding buffer containing NP40 followed by centrifugation and resuspension in binding buffer). Imidazole was removed by washing the resin with 50 mM NaHPO₄, 0.3M NaCl (pH 7.0). Protein bound to the resin was eluted using the same buffer (50 mM NaHPO₄, 0.3M NaCl) having a reduced pH (pH 3.5-4.0).

A pilot purification was performed following this protocol and yielded 18 mg/liter affinity-purified pHisBot. The pHisBot protein was greater than 90% pure as estimated by Coomassie staining of an SDS-PAGE gel. This represents the highest observed yield of soluble affinity-purified pHisBot protein and this protocol eliminates the need for separate imidazole-containing binding and wash buffers. In addition to providing a simplified and efficient protocol for the affinity purification of recombinant pHisBot protein, the above results provide a variety of purification conditions under which pHisBot protein can be isolated.

EXAMPLE 26

The pHisBot Protein Is An Effective Immunogen

In Example 23 it was demonstrated that neutralizing antibodies are generated in mouse serum after nasal immunization with the pMBot protein. However, the pMBot protein was found to copurify with significant amounts of endotoxin which could not be easily removed. The pHisBot protein, in contrast, could be isolated free of significant endotoxin contamination making pHisBot a superior candidate for vaccine production. To further assess the suitability of pHisBot as a vaccine, the immunogenicity of the pHisBot protein was determined and a comparison of the relative immunogenicity of pMBot and pHisBot proteins in mice was performed as follows.

Two groups of eight BALBc mice were immunized with either pMBot protein or pHisBot protein using Gerbu GMDP adjuvant (CC Biotech). pMBot protein (in PBS containing 10 mM maltose) or pHisBot protein (in 50 mMNaHPO₄, 0.3 M NaCl, 10% glycerol, pH 4.0) was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received an IP injection of 100 µl antigen/adjuvant mix (50 µg antigen plus 1 µg adjuvant) on day 0. Mice were boosted as described above with the exception that the route of administration was IM on day 14 and 28. The mice were bled on day 77 and anti-*C. botulinum* Type A toxoid titers were determined using serum collected from individual mice in each group (as described in Example 23). The results are shown in Table 41:

TABLE 41

Anti-*C. botulinum* Type A Toxoid Serum IgG Titers In Individual Mice Immunized With pMBot or pHisBot Protein

Mouse #	Preimmune ¹				pMBot ²				pHisBot ²			
	Sample Dilution				Sample Dilution				Sample Dilution			
	1:50	1:250	1:1250	1:6250	1:50	1:250	1:1250	1:6250	1:50	1:250	1:1250	1:620
1					0.678	0.190	0.055	0.007	1.574	0.799	0.320	0.093
2					1.161	0.931	0.254	0.075	1.513	0.829	0.409	0.134
3					1.364	0.458	0.195	0.041	1.596	1.028	0.453	0.122
4					1.622	1.189	0.334	0.067	1.552	0.840	0.348	0.090
5					1.612	1.030	0.289	0.067	1.629	1.580	0.895	0.233
6					0.913	0.242	0.069	0.013	1.485	0.952	0.477	0.145
7					0.910	0.235	0.058	0.014	1.524	0.725	0.269	0.069
8					0.747	0.234	0.058	0.014	1.274	0.427	0.116	0.029
Mean Titer	0.048	0.021	0.011	0.002	1.133	0.564	0.164	0.037	1.518	0.896	0.411	0.114

¹ The preimmune sample represents the average from 2 sets of duplicate wells containing serum from a individual mouse immunized with recombinant *Staphylococcus enterotoxin B* (SEB) antigen. This antigen is immunologically unrelated to *C. botulinum* toxin and provides a control serum.

² Average of duplicate wells.

The results shown above in Table 41 demonstrate that both the pMBot and pHisBot proteins are immunogenic in mice as 100% of the mice (8/8) in each group seroconverted from non-immune to immune status. The results also show that the average titer of anti-*C. botulinum* Type A toxoid IgG is 2-3 fold higher after immunization with the pHisBot protein relative to immunization with the pMBot protein. This suggests that the pHisBot protein may be a superior immunogen to the pMBot protein.

EXAMPLE 27

Immunization With The Recombinant pHisBot Protein Generates Neutralizing Antibodies

The results shown in Example 26 demonstrated that both the pHisBot and pMBot proteins were capable of inducing high titers of anti-*C. botulinum* type A toxoid-reactive antibodies in immunized hosts. The ability of the immune sera from mice immunized with either the pHisBot or pMBot proteins to neutralize *C. botulinum* type A toxoid *in vivo* was determined using the mouse neutralization assay described in Example 23b.

The two groups of eight BALBc mice immunized with either pMBot protein or pHisBot protein in Example 26 were boosted again one week after the bleeding on day 77. The boost was performed by mixing pMBot protein (in PBS containing 10 mM maltose) or pHisBot protein (in 50 mM NaHPO₄, 0.3 M NaCl, 10% glycerol, pH 4.0) with Gerbu adjuvant as described in Example 26. Each mouse received an IP injection of 100 µl antigen/adjuvant mix (50 µg antigen plus 1 µg adjuvant). The mice were bled 6 days after this boost and the serum from mice within a group was pooled. Serum from preimmune mice was also collected (this serum is the same serum described in the footnote to Table 41).

The presence of neutralizing antibodies in the pooled or preimmune serum was detected by challenging mice with 5 LD₅₀ units of type A toxin mixed with 100 µl of pooled serum. The challenge was performed by mixing (per mouse to be injected) 100 µl of serum from each pool with 100 µl of purified type A toxin standard (50 LD₅₀/ml prepared as described in Example 23b) and 500 µl of gel-phosphate. The mixtures were incubated for 30 min at room temperature with occasional mixing. Each of four mice were injected IP with the mixtures (0.7 ml/mouse). The mice were observed for signs of botulism for 72 hours. Mice receiving toxin mixed with serum from mice immunized with either the pHisBot or pMBot proteins showed no signs of botulism intoxication. In contrast, mice receiving preimmune serum died in less than 24 hours.

These results demonstrate that antibodies capable of neutralizing *C. botulinum* type A toxin are induced when either of the recombinant *C. botulinum* C fragment proteins pHisBot or pMBot are used as immunogens.

EXAMPLE 28

Cloning And Expression Of The C Fragment of *C. botulinum* Serotype A Toxin In *E. coli* Utilizing A Native Gene Fragment

In Example 22 above, a synthetic gene was used to express the C fragment of *C. botulinum* serotype A toxin in *E. coli*. The synthetic gene replaced non-preferred (*i.e.*, rare) codons present in the C fragment gene with codons which are preferred by *E. coli*. The synthetic gene was generated because it was been reported that genes which have a high A/T content (such as most clostridial genes) creates expression difficulties in *E. coli* and yeast. Furthermore, LaPenotiere *et al.* suggested that problems encountered with the stability (non-fusion constructs) and solubility (MBP fusion constructs) of the C fragment of *C. botulinum* serotype A toxin when expressed in *E. coli* was most likely due to the extreme A/T richness of the native *C. botulinum* serotype A toxin gene sequences (LaPenotiere, *et al.*, *supra*).

In this example, it was demonstrated that successful expression of the C fragment of *C. botulinum* type A toxin gene in *E. coli* does not require the elimination of rare codons (*i.e.*, there is no need to use a synthetic gene). This example involved a) the cloning of the native C fragment of the *C. botulinum* serotype A toxin gene and construction of an expression vector and b) a comparison of the expression and purification yields of *C. botulinum* serotype A C fragments derived from native and synthetic expression vectors.

a) **Cloning Of The Native C Fragment Of The
C. botulinum Serotype A Toxin Gene And
Construction Of An Expression Vector**

The serotype A toxin gene was cloned from *C. botulinum* genomic DNA using PCR amplification. The following primer pair was employed: 5'-CGCCATGGCTAG ATTATTATCTACATTTAC-3' (5' primer, *Nco*I site underlined; SEQ ID NO:29) and 5'-GCAAGCTTCTTGACAGACTCATGTAG-3' (3' primer, *Hind*III site underlined; SEQ ID NO:30). *C. botulinum* type A strain was obtained from the American Type Culture Collection (ATCC#19397) and grown under anaerobic conditions in Terrific broth medium. High molecular-weight *C. botulinum* DNA was isolated as described in Example 11. The integrity and yield of genomic DNA was assessed by comparison with a serial dilution of uncut lambda DNA after electrophoresis on an agarose gel.

The gene fragment was cloned by PCR utilizing a proofreading thermostable DNA polymerase (native *Pfu* polymerase). PCR amplification was performed using the above primer pair in a 50 μ l reaction containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM MgCl₂, 200 μ M each dNTP, 0.2 μ M each primer, and 50ng *C. botulinum* genomic DNA. Reactions were overlaid with 100 μ l mineral oil, heated to 94°C 4 min, 0.5 μ l native *Pfu* polymerase (Stratagene) was added, and thirty cycles comprising 94°C for 1 min, 50°C for 2 min, 72°C for 2 min were carried out followed by 10 min at 72°C. An aliquot (10 μ l) of the reaction mixture was resolved on an agarose gel and the amplified native C fragment gene was gel purified using the Prep-A-Gene kit (BioRad) and ligated to pCRScript vector DNA (Stratagene). Recombinant clones were isolated and confirmed by restriction digestion, using standard recombinant molecular biology techniques [Sambrook *et al.* (1989), *supra*]. In addition, the sequence of approximately 300 bases located at the 5' end of the C fragment coding region were obtained using standard DNA sequencing methods. The sequence obtained was identical to that of the published sequence.

An expression vector containing the native *C. botulinum* serotype A C fragment gene was created by ligation of the *Nco*I-*Hind*III fragment containing the C fragment

gene from the pCRScript clone to *NheI*-*HindIII* restricted pETHisa vector (Example 18b). The *NcoI* and *NheI* sites were filled in using the Klenow enzyme prior to ligation; these sites were thus blunt-end ligated together. The resulting construct was termed pHisBotA (native). pHisBotA (native) expresses the *C. botulinum* serotype A C fragment with a his-tagged N terminal extension which has the following sequence: MetGlyHisHisHisHisHisHisHisHisHisSerSerGlyHis*lleGluGlyArgHisMetAla* (SEQ ID NO:24), where the underlining represents amino acids encoded by the *C. botulinum* C fragment gene (this N terminal extension contains the recognition site for FactorXa protease, shown in italics, which can be employed to removed the polyhistidine tract from the N-terminus of the fusion protein). The pHisBot (native) construct expresses the identical protein as the pHisBot construct (Ex. 24c; herein after the pHisBotA) which contains the synthetic gene.

The predicted DNA sequence encoding the native *C. botulinum* serotype A C fragment gene contained within pHisBotA (native) is listed in SEQ ID NO:31 [the start of translation (ATG) is located at nucleotides 108-110 and the stop of translation (TAA) is located at nucleotides 1494-1496 in SEQ ID NO:31] and the corresponding amino acid sequence is listed in SEQ ID NO:26 (*i.e.*, the same amino acid sequence as that produced by pHisBotA containing synthetic gene sequences).

**b) Comparison Of The Expression And Purification
Yields Of *C. botulinum* Serotype A C Fragments
Derived From Native And Synthetic Expression
Vectors**

Recombinant plasmids containing either the native or the synthetic *C. botulinum* serotype A C fragment genes were transformed into *E. coli* strain BI21(DE3) pLysS and protein expression was induced in 1 liter shaker flask cultures. Total protein extracts were isolated, resolved on SDS-PAGE gels and *C. botulinum* C fragment protein was identified by Western analysis utilizing a chicken anti-*C. botulinum* serotype A toxoid antiserum as described in Example 22.

Briefly, 1 liter (2XYT + 100 µg/ml ampicillin and 34 µg/ml chloramphenicol) cultures of bacteria harboring either the pHisBotA (synthetic) or pHisBotA (native) plasmids in the BL21(DE3) pLysS strain were induced to express recombinant protein by addition of IPTG to 1mM. Cultures were grown at 30-32°C, IPTG was added
5 when the cell density reached an OD₆₀₀ 0.5-1.0 and the induced protein was allowed to accumulate for 3-4 hrs after induction.

The cells were cooled for 15 min in a ice water bath and then centrifuged for 10 min at 5000 rpm in a JA10 rotor (Beckman) at 4°C. The cell pellets were resuspended in a total volume of 40 mls 1X binding buffer (40 mM imidazole, 0.5 M
10 NaCl, 50 mM NaPO₄, pH 8.0), transferred to two 50 ml Oakridge tubes and frozen at -70°C for at least 1 hr. The tubes were then thawed and the cells were lysed by sonication (using four successive 20 second bursts) on ice. The suspension was clarified by centrifugation 20-30 min at 9,000 rpm (10,000g) in a JA-17 rotor. The soluble lysate was batch absorbed to 7 ml of a 1:1 slurry of NiNTA resin:binding
15 buffer by stirring 2-4 hr at 4°C. The slurry was centrifuged for 1 min at 500g in 50 ml tube (Falcon), resuspended in 5 mls binding buffer and poured into a 2.5 cm diameter column (BioRad). The column was attached to a UV monitor (ISCO) and the column was washed with binding buffer until a baseline was established. Imidazole was removed by washing with 50mM NaPO₄, 0.3 M NaCl, 10% glycerol,
20 pH 7.0 and bound protein was eluted using 50mM NaPO₄, 0.3 M NaCl, 10% glycerol, pH 3.5-4.0.

The eluted proteins were stored at 4°C. Samples of total, soluble, and eluted proteins were resolved by SDS-PAGE. Protein samples were prepared for electrophoresis by mixing 1µl total (T) or soluble (S) protein with 4 µl PBS and 5 µl
25 2X SDS-PAGE sample buffer, or 5 µl eluted (E) protein and 5 µl 2X SDS-PAGE sample buffer. The samples were heated to 95°C for 5 min, then cooled and 5 or 10 µls were loaded on 12.5% SDS-PAGE gels. Broad range molecular weight protein markers (BioRad) were also loaded to allow the MW of the identified fusion proteins to be estimated. After electrophoresis, protein was detected either generally by

staining gels with Coomassie blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein.

For Western blot analysis, the gels were blotted, and protein transfer was confirmed by Ponceau S staining as described in Example 22. After blocking the blots for 1 hr at room temperature in blocking buffer (PBST and 5% milk), 10 ml of a 1/500 dilution of an anti-*C. botulinum* toxin A IgY PEG prep (Ex. 3) in blocking buffer was added and the blots were incubated for an additional hour at room temperature. The blots were washed and developed using a rabbit anti-chicken alkaline phosphatase conjugate (Boehringer Mannheim) as the secondary antibody as described in Ex. 22. This analysis detected *C. botulinum* toxin A-reactive proteins in the pHisBotA (native and synthetic) protein samples (corresponding to the predicted full length proteins identified by Coomassie staining).

A gel containing proteins expressed from the pHisBot and pHisBot (native) constructs during various stages of purification and stained with Coomassie blue is shown in Figure 31. In Figure 31, lanes 1-4 and 9 contain proteins expressed by the pHisBotA construct (*i.e.*, the synthetic gene) and lanes 5-8 contain proteins expressed by the pHisBotA (native) construct. Lanes 1 and 5 contain total protein extracts; lanes 2 and 6 contain soluble protein extracts; lanes 3 and 7 contain proteins which flowed through the NiNTA columns; lanes 4, 8 and 9 contain protein eluted from the NiNTA columns and lane 10 contains molecular weight markers.

The above purification resulted in a yield of 3 mg (native gene) or 11 mg (synthetic gene) of affinity purified protein from a 1 liter starting culture, of which at least 90-95% of the protein was a single band of the predicted MW (50kd) and immunoreactivity for recombinant *C. botulinum* serotype A C fragment protein. Other than the level of expression, no difference was observed between the native and the synthetic gene expression systems.

These results demonstrate that soluble *C. botulinum* serotype A C fragment protein can be expressed in *E. coli* and purified utilizing either native or synthetic gene sequences.

EXAMPLE 29

Generation Of Neutralizing Antibodies Using A Recombinant *C. botulinum* Serotype A C Fragment Protein Containing A Six Residue His-Tag

In Example 27, neutralizing antibodies were generated utilizing the pHisBotA protein, which contains a histidine-tagged N-terminal extension comprising 10 histidine residues. To determine if the generation of neutralizing antibodies is dependent on the presence of this particular his-tag, a protein containing a shorter N-terminal extension (comprising 6 histidine residues) was produced and tested for the ability to generate neutralizing antibodies. This example involved a) the cloning and expression of the p6HisBotA(syn) protein and b) the generation and characterization of hyperimmune serum.

a) Cloning And Expression Of The p6HisBotA(syn) Protein

The p6HisBotA(syn) construct was generated as described below; the term "syn" designates the presence of synthetic gene sequences. This construct expresses the C fragment of the *C. botulinum* serotype A toxin with a histidine-tagged N terminal extension having the following sequence: MetHisHisHisHisHisHisMetAla (SEQ ID NO:32); the amino acids encoded by the botulinal C fragment gene are underlined and the vector encoded amino acids are presented in plain type.

6XHis oligonucleotides [5'-TATGCATCACCATCACCATCA-3' (SEQ ID NO:33) and 5'-CATGTGATGGTGATGGTGATGCA-3' (SEQ ID NO:34) were annealed as follows. One microgram of each oligonucleotide was mixed in total of 20 μ l 1X reaction buffer 2 (NEB) and the mixture was heated at 70°C for 5 min and then incubated at 42°C for 5 min. The annealed oligonucleotides were then ligated with gel purified *Nde*I/*Hind*III cleaved pET23b (T7 promoter) or pET21b (T7lac promoter) DNA and the gel purified *Nco*I/*Hind*III *C. botulinum* serotype A C fragment synthetic gene fragment derived from pAlterBot (Ex. 22). Recombinant clones were isolated and confirmed by restriction digestion. The DNA sequence encoding the 6X his-

tagged BotA protein contained within p6HisBotA(syn) is listed in SEQ ID NO:35.
The amino acid sequence of the p6XHisBotA protein is listed in SEQ ID NO:36.

The resulting recombinant p6XHisBotA plasmid was transformed into the BL21(DE3) pLysS strain, and 1 liter cultures were grown, induced and harvested as described in Example 28. His-tagged protein was purified as described in Example 28, with the following modifications. The binding buffer (BB) contained 5 mM imidazole rather than 40 mM imidazole and NP40 was added to the soluble lysate to a final concentration of 0.1%. The bound material was washed on the column with BB until the baseline was established, then the column was washed successively with BB+20 mM imidazole and BB+40 mM imidazole. The column was eluted as described in Example 28.

In the case of the pET23-derived expression system, high level expression of insoluble 6HisBotA protein was induced. The pET21-derived vector expressed lower levels of soluble protein that bound the NiNTA resin and eluted in the 40 mM imidazole wash rather than during the low pH elution. These results (*i.e.*, low level expression of a soluble protein) are consistent with the results obtained with pHisBotA protein (Ex. 25); the pHisBotA construct, like the pET21-derived vector, contains the T7lac rather than T7 promoter.

The 6HisBotA protein thus elutes under less stringent conditions than the 10X histidine-containing pHisBot protein (100-200 mM imidazole; Ex. 25) presumably due to the reduction in the length of the his-tag. The eluted protein was of the predicted size [*i.e.*, slightly reduced in comparison to pHisBotA protein].

b) Generation And Characterization Of Hyperimmune Serum

Eight BALBc mice were immunized with purified 6HisBotA protein using Gerbu GMDP adjuvant (CC Biotech). The 40 mM imidazole elution was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received a subcutaneous injection of 100 μ l antigen/adjuvant mix (12 μ g antigen + 1 μ g adjuvant) on day 0. Mice were subcutaneously boosted as above on day 14 and bled on day 28. Control

mice received pHisBotB protein (prepared as described in Ex. 35 below) in Gerbu adjuvant.

Anti-*C. botulinum* serotype A toxoid titers were determined in serum from individual mice from each group using the ELISA described in Example 23a with the exception that the initial testing serum dilution was 1:100 in blocking buffer containing 0.5% Tween 20, followed by serial 5-fold dilutions into this buffer. The results of the ELISA demonstrated that seroconversion (relative to control mice) occurred in all 8 mice.

The ability of the anti-*C. botulinum* serotype A C fragment antibodies present in serum from the immunized mice to neutralize native *C. botulinum* type A toxin was tested using the mouse neutralization assay described in Example 23b. The amount of neutralizing antibodies present in the serum of the immunized mice was determined using serum antibody titrations. The various serum dilutions (0.01 ml) were mixed with 5 LD₅₀ units of *C. botulinum* type A toxin and the mixtures were injected IP into mice. The neutralizations were performed in duplicate. The mice were then observed for signs of botulism for 4 days. Undiluted serum was found to protect 100% of the injected mice while the 1:10 diluted serum did not. This corresponds to a neutralization titer of 0.05-0.5 IU/ml.

These results demonstrate that neutralizing antibodies were induced when the 6HisBotA protein was utilized as the immunogen. Furthermore, these results demonstrate that seroconversion and the generation of neutralizing antibodies does not depend on the specific N terminal extension present on the recombinant *C. botulinum* type A C fragment proteins.

EXAMPLE 30

Construction Of Vectors For The Expression Of His-Tagged *C. botulinum* Type A Toxin C Fragment Protein Using the Synthetic Gene

A number of expression vectors were constructed which contained the synthetic
5 *C. botulinum* type A toxin C fragment gene. These constructs vary as to the promoter
(T7 or T7lac) and repressor elements (lacIq) present on the plasmid. The T7 promoter
is a stronger promoter than is the T7lac promoter. The various constructs provide
varying expression levels and varying levels of plasmid stability. This example
involved a) the construction of expression vectors containing the synthetic *C.*
10 *botulinum* type A C fragment gene and b) the determination of the expression level
achieved using plasmids containing either the kanamycin resistance or the ampicillin
resistance genes in small scale cultures.

a) Construction Of Expression Vectors Containing The Synthetic *C. botulinum* Type A C Fragment Gene

15 Expression vectors containing the synthetic *C. botulinum* type A C fragment
gene were engineered to utilize the kanamycin resistance rather than the ampicillin
resistance gene. This was done for several reasons including concerns regarding the
presence of residual ampicillin in recombinant protein derived from plasmids
containing the ampicillin resistance gene. In addition, ampicillin resistant plasmids are
20 more difficult to maintain in culture; the β -lactamase secreted by cells containing
ampicillin resistant plasmids rapidly degrades extracellular ampicillin, allowing the
growth of plasmid-negative cells.

A second altered feature of the expression vectors is the inclusion of lacIq gene
in the plasmid. This repressor lowers expression from lac regulated promoters (the
25 chromosomally located, lactose regulated T7 polymerase gene and the plasmid located
T7lac promoter). This down regulates uninduced protein expression and can enhance
the stability of recombinant cell lines. The final alteration to the vectors is the

inclusion of either the T7 or T7lac promoters that drive high or moderate level expression of recombinant protein, respectively.

5 The expression plasmids were constructed as follows. In all cases, the protein expressed is the pHisBotA(syn) protein previously described, and the only differences between constructs is the alteration of the various regulatory elements described above.

i) **Construction Of pHisBotA(syn) kan
T7lac**

10 The pHisBotA(syn) kan T7lac construct was made by inserting the *SapI/XhoI* fragment containing the *C. botulinum* type A C fragment from pHisBotA(syn) into pET24 digested with *SapI/XhoI* (Novagen; fragment contains kan gene and origin of replication). The desired construct was selected for kanamycin resistance and confirmed by restriction digestion.

ii) **Construction Of pHisBotA(syn) kan
lacIq T7lac**

15 The pHisBotA(syn) kan lacIq T7lac construct was made by inserting the *XbaI/HindIII* fragment containing the *C. botulinum* type A C fragment from pHisBotA(syn)kanT7lac into the pET24a vector digested with *XbaI/HindIII*. The resulting construct was confirmed by restriction digestion.

20 iii) **Construction Of pHisBotA(syn) kan
lacIq T7**

25 The pHisBotA(syn) kan lacIq T7 construct was made by inserting the *XbaI/HindIII* fragment containing the *C. botulinum* type A C fragment from pHisBotA(syn) kan lacIq T7lac into *XbaI/HindIII*-digested pHisBotB(syn) kan lacIq T7 (described in Ex 37c below). The resulting construct was confirmed by restriction digestion.

**b) Determination Of The Expression Level Achieved
Using Plasmids Containing Either The Kanamycin
Resistance Or The Ampicillin Resistance Genes In
Small Scale Cultures**

5 One liter cultures of pHisBotA(syn) kan T7lac/BI21(DE3)pLysS and
pHisBotA(syn) amp T7lac/BI21(DE3)pLysS [this is the previously designated
pHisBotA(syn) construct] were grown, induced and his-tagged proteins were purified
as described in Example 28. No differences in yield or protein integrity/purity were
observed.

10 These results demonstrate that the antigen induction levels from expression
constructs were not affected by the choice of ampicillin versus kanamycin antibiotic
resistance genes.

EXAMPLE 31

Fermentation Of Cells Expressing Recombinant Botulinal Proteins

15 **a) Fermentation Culture Of Cells Expressing
Recombinant Botulinal Proteins**

Fermentation cultures were grown under the following conditions which were
optimized for growth of the BL21(DE3) strains containing pET derived expression
vectors. An overnight 1 liter feeder culture was prepared by inoculating of 1 liter
20 media (in a 2L shaker flask) with a fresh colony grown on an LB kan plate. The
feeder culture contained: 600 mls nitrogen source [20 gm yeast extract (BBL) and 40
gm tryptone (BBL)/600 mls], 200 mls 5X fermentation salts (per liter: 48.5 gm
K₂HPO₄, 12 gm NaH₂PO₄•H₂O, 5 gm NH₄Cl, 2.5 gm NaCl), 180 mls dH₂O, 20 mls
20% glucose, 2 mls 1 M MgSO₄, 5 mls 0.05M CaCl₂ and 4 mls of a 10 mg/ml
25 kanamycin stock. All solutions were sterilized by autoclaving, except the kanamycin
stock which was filter sterilized.

An aliquot (5 ml) of the feeder culture broth was removed prior to inoculation, and grown for 2 days at 37°C as a culture broth sterility control. Growth was not observed in this control culture in any of the fermentations performed.

The inoculated feeder culture was grown for 12-15 hrs (ON) at 30-37°C. Care was taken to prevent oversaturation of this culture. The saturated feeder culture was added to 10L of fermentation media in fermenter (BiofloIV, New Brunswick Scientific, Edison, NJ) as follows. The fermenter was sterilized 120 min at 121°C with dH₂O. The sterile water was removed, and fermentation media added as follows: 6 liters nitrogen source, 2 liters 5X fermentation salts, 2 liters 2% glucose, 20 mls 1 M MgSO₄, 50 mls 0.05 M CaCl₂, 2.5-3.5 mls Macol P 400 antifoam (PPG Industries Inc., Gurnee, IL), 40 mls 10mg/ml kanamycin and 10 mls trace elements (8 gm FeSO₄•7H₂O, 2 gm MnSO₄•H₂O, 2 gm AlCl₃•6H₂O, 0.8 gm CoCl₂•6H₂O, 0.4 gm ZnSO₄•7H₂O, 0.4 gm Na₂MoO₄•2H₂O, 0.2 gm CuCl₂•2H₂O, 0.2 gm NiCl₂, 0.1 gm H₃BO₃/200mls 5 M HCl). All solutions were sterilized by autoclaving, except the kanamycin stock which was filter sterilized. Fermentation media was prewarmed to 37°C before the addition of the feeder culture.

After the addition of the feeder culture, the culture was fermented at 37°C, 400 rpm agitation, and 10 l/min air sparging. The DO₂ control was set to 20% PID and dissolved oxygen levels were controlled by increasing the rate of agitation from 400-850 rpm under DO₂ control. DO₂ levels were maintained at greater than or equal to 20% throughout the entire fermentation. When agitation levels reached 500-600 rpm the temperature was lowered to 30°C to reduce the oxygen consumption rate. Culture growth was continued until endogenous carbon sources were depleted. In these fermentations, glucose was depleted first [monitored with a glucose monitoring kit (Sigma)], followed by assimilation of acetate and other acidic carbons [monitored using an acetate test kit (Boehringer Mannheim)]. During the assimilation phase, the pH rose from 6.6-6.8 (starting pH) to 7.4-7.5, at which time the bulk of the remaining carbon source was depleted. This was signaled by a drop in agitation rate (from a maximum of 700-800 rpm) and a rise in DO₂ levels >30%. This corresponds to a OD₆₀₀ reading of 18-20/ml. At this point a fed batch mode was initiated, in which a

feed solution of 50% glucose was added at a rate of approximately 4 gm glucose/liter/hr. The pH was adjusted to 7.0 by the addition of 25% H₃PO₄ (approximately 60 mls). Culture growth was continued and reached peak oxygen consumption within the next 3 hrs of growth (while the remaining residual non-glucose carbon sources were assimilated). This phase is characterized by a slow increase in pH, and air sparging was increased to 15L/min, to keep the maximum rpm below 850.

Once the residual acidic carbon sources are depleted the agitation rate decreases to 650-750 rpm and the pH begins to drop. pH control was maintained at 7.0 PID by regulated pump addition of a sterile 4M NaOH solution which was consumed at a steady rate for the remainder of the fermentation. Growth was continued at 30°C, and the cultures were grown linearly at a growth rate of 4-7 OD₆₀₀ units/hr, to at least 81.5 OD₆₀₀ units/ml (>30g/l dry cell weight) without induction. Antifoam (a 1:1 dilution with filter sterilized 100% ethanol) was added as necessary throughout the fermentation to prevent foaming.

During the fed batch mode, glucose was assimilated immediately (concentration in media consistently less than 0.1 gm/liter) and acetate was not produced in significant levels by the pET plasmid/BL21(DE3) cell lines tested (approximately 1 gm/liter at end of fermentation; this is lower than that observed in harvests from shaker flask cultures utilizing the same strains). This was fortuitous, since high levels of acetate has been shown to inhibit induction levels in a variety of expression systems. The above described conditions were found to be highly reproducible between fermentations and utilizing different expression plasmids. As a result, glucose and acetate level monitoring were no longer preformed during fermentation.

b) Induction Of Fermentation Cultures

Induction with IPTG (250 mg-10 gms, depending on the expression vector and experiment) was initiated 1-3 hrs after initiation of the glucose feed (30-50 OD₆₀₀/ml). The growth rate after induction was monitored on a hourly basis. Aliquots (5-10 ml) of cells were harvested at the time of induction, and at hourly intervals post-induction. Optical density readings were determined by measuring the absorbance at 600 nm of

10 µl culture in 990 µl PBS versus a PBS control. The growth rate after induction was found to vary depending on the expression system utilized.

c) Monitoring Of Fermentation Cultures

Fermentation cultures were monitored using the following control assays.

5 **i) Colony Forming Ability**

An aliquots of cells were removed from the cultures at each timepoint sampled (uninduced and at various times after induction) were serially diluted in PBS (dilution 1=15 µl cells/3 ml PBS, dilution 2 = 15 µl of dilution 1/3 ml PBS, dilution 3 = 3 or 6 µl of dilution 2/3mls PBS) and 100 µl of dilution 3 was plated on an LB or TSA
10 (trypticase soy agar) plate. The plates were incubated ON at 37°C and then the colonies are counted and scored for macro or micro growth.

ii) Phenotypic Characterization

Colonies growing on LB or TSA plates (above) from uninduced and induced timepoints were replica plated onto LB+kan, LB+chloramphenicol (for fermentations
15 utilizing LysS or pACYCGro plasmids), LB+kan+1mM IPTG and LB plates, in this order. The plates were grown 6-8 hrs at 37°C and growth was scored on each plate for a minimum of 40-50 well isolated colonies. The percentage of cells retaining the plasmid at time of induction (*i.e.*, uninduced cultures immediately prior to the addition of IPTG) was determined to be the # colonies LB+Kan (or chloramphenicol) plate/#
20 colonies LB plate X 100%. The percentage of cells with mutated pET plasmids was determined to be the # colonies LB+Kan+IPTG plate/# colonies LB plate X 100%. Colonies on all LB plates were scored morphologically for *E. coli* phenotype as a contamination control. Morphologically detectable contaminant colonies were not detected in any fermentation.

iii) Recombinant BotA Protein Induction

A total of 10 OD₆₀₀ units of cells (*e.g.*, 200 µl of cells at OD₆₀₀=50/ml) were removed from each timepoint sample to a 1.5 ml microfuge tube and pelleted for 2 min at maximum rpm in a microfuge. The pellets were resuspended in 1 ml of 50 mM NaHPO₄, 0.5 M NaCl, 40mM imidazole buffer (pH 6.8) containing 1 mg/ml lysozyme. The samples were incubated for 20 min at room temperature and stored ON at -70°C. Samples were thawed completely at room temperature and sonicated 2 X 10 seconds with a Branson Sonifier 450 microtip probe at # 3 power setting. The samples were centrifuged for 5 min at maximum rpm in a microfuge.

An aliquot (20 µl) of the protein samples were removed to 20 µl 2X sample buffer, before or after centrifugation, for total and soluble protein extracts, respectively. The samples were heated to 95°C for 5 min, then cooled and 5 or 10 µl were loaded onto 12.5% SDS-PAGE gels. High molecular weight protein markers (BioRad) were also loaded to allow for estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining gels with Coomassie blue, or specifically, by blotting onto nitrocellulose (as described in Ex. 28) for Western blot detection of specific his-tagged proteins utilizing a NiNTA-alkaline phosphatase conjugate exactly as described by the manufacturer (Qiagen).

iv) Recombinant Antigen Purification

At the end of each fermentation run, 1-10 liters of culture were harvested from the fermenter and the bacterial cells were pelleted by centrifugation at 6000 rpm for 10 min in a JA10 rotor (Beckman). The cell pellets were stored frozen at -70°C or utilized immediately without freezing. Cell pellets were resuspended to 15-20% weight to volume in resuspension buffer (generally 50 mM NaPO₄, 0.5 M NaCl, 40mM imidazole, pH 6.8) and lysed utilizing either sonication or high pressure homogenization.

For sonication, the resuspension buffer was supplemented with lysozyme to 1 mg/ml, and the suspension was incubated for 20 min. at room temp. The sample was

then frozen ON at -70°C, thawed and sonicated 4 X 20 seconds at microtip maximum to reduce viscosity. For homogenization, the cells were lyzed by 2 passes through a homogenizer (Rannie Mini-lab type 8.30 H) at 600 Bar. Cell lysates were clarified by centrifugation for 30 min at 10,000 rpm in a JA10 rotor.

5 For IDA chromatography, samples were flocculated utilizing polyethyleneimine (PEI) prior to centrifugation. Cell pellets were resuspended in cell resuspension buffer (CRB: 50 mM NaPO₄, 0.5 M NaCl, 40 mM imidazole, pH 6.8) to create a 20% cell suspension (wet weight of cells/volume of CRB) and cell lysates were prepared as described above (sonication or homogenization). PEI (a 2% solution in dH₂O, pH 7.5
10 with HCl) was added to the cell lysate a final concentration of 0.2%, and stirred for 20 min at room temperature prior to centrifugation (8,500 rpm in JA10 rotor for 30 minutes at 4°C). This treatment removed RNA, DNA and cell wall components, resulting in a clarified, low viscosity lysate ("PEI clarified lysate").

His-tagged proteins were purified from soluble lysates by metal-chelate affinity
15 chromatography using either a NiNTA resin (as described in Ex. 28) or an IDA (iminodiacetic acid) resin as described below.

IDA resin affinity purifications were performed utilizing a low pressure chromatography system (ISCO). A 7 ml (small scale) or 70 ml (large scale) Chelating Sepharose Fast Flow (Pharmacia) affinity column was poured; in addition, a second
20 guard column was poured and attached in line with the first column (to capture Ni ions that leached off the affinity column). The columns were washed with 3 column volumes of dH₂O. The guard column was then removed and the affinity column was washed with 0.3 M NiSO₄ until resistivity was established, then with dH₂O until the resistivity returned to baseline. The columns were reconnected and equilibrated with
25 cell resuspension buffer (CRB; 50 mM NaPO₄, 0.5 M NaCl, 40 mM imidazole, pH 6.8). The clarified sample (in CRB) was loaded. Flow rates were 5 ml/min for small scale columns and 20 ml/min for large scale columns. After sample loading, the column was washed with CRB until a baseline established and bound protein was eluted with elution buffer (50 mM NaPO₄, 0.5 M NaCl, 800 mM imidazole, 20%
30 glycerol, pH 6.8 or 8.0). Protein samples were stored at 4°C or -20°C. The yield of

eluted protein was established by measuring the OD₂₈₀ of the elutions, with a 1 mg/ml solution of protein assumed to yield an absorbance reading of 2.0.

The IDA columns may be regenerated and reused multiple times (>10). To regenerate the column, the column was washed with 2-3 column volumes of H₂O, then 0.05 M EDTA until all of the blue/green color was removed followed by a wash with dH₂O. The IDA columns were sterilized with 0.1 M NaOH (using at least 3 column volumes but not more than 50 minutes contact time with column packing material), then washed with 3 column volumes 0.05 M NaPO₄, pH 5.0, then dH₂O and stored at room temperature in 20 % ethanol.

EXAMPLE 32

Construction Of A Folding Chaperone Overexpression System

Co-overexpression of the *E. coli* GroEL/GroES folding chaperones in a cell expressing a recombinant foreign protein has been reported to enhance the solubility of some foreign proteins that are otherwise insoluble when expressed in *E. coli* [Gragerouu *et al.* (1992) Proc. Natl. Acad. Sci. USA 89:10344]. The improvement in solubility is thought to be due to chaperone-mediated binding and unfolding of insoluble denatured proteins, thus allowing multiple attempts for productive refolding of recombinant proteins. By overexpressing the chaperones, the unfolding/refolding reaction is driven by excess chaperone, resulting, in some cases, in higher yields of soluble protein.

In this example, a chaperone overexpression system, compatible with pET vector expression systems, was constructed to facilitate testing chaperone-mediated solubilization of *C. botulinum* type A proteins. This example involved the cloning of the GroEL/ES operon and construction of a pLysS-based chaperone hyperexpression system.

The GroEL/GroES operon was PCR amplified and cloned into the pCRScript vector as described in Example 28. The following primer pair was used: 5'-CGCAT ATGAATATTCGTCCATTGCATG-3' (SEQ ID NO:37) [5' primer, start codon of

groES gene converted to *Nde*I site (underlined)] and 5'-GGAAGCTTGCAGGGCAAT
TACATCATG (SEQ ID NO:38) (3' primer, stop codon of groEL gene italicized,
engineered *Hind*III site underlined). Following amplification, the chaperone operon
was excised as an *Nde*I/*Hind*III fragment and cloned into pET23b digested with *Nde*I
5 and *Hind*III. This construction places the Gro operon under the control of the T7
promoter of the pET23 vector. The desired construct was confirmed by restriction
digestion.

The T7 promoter-Gro operon-T7 terminator expression cassette was then
excised as a *Bgl*II/*Bsp*EI (filled) fragment and cloned into *Bam*HI (compatible with
10 *Bgl*II)/*Hind*III (filled) cleaved pLysS plasmid (this removed the T7 lysozyme gene).
The resulting construct was designated pACYCGro, since the plasmid utilizing the
pACYC184 origin from the plysS plasmid. Proper construction was confirmed by
restriction digestion.

pACYCGro was transformed into BL21(DE3), cultures were grown and
15 induced with 1 mM IPTG as described in preceding examples. Total and soluble
protein extracts were generated from cells removed before and after IPTG induction
and were resolved on a 12.5 % SDS-PAGE gel and stained with Coomassie blue.
This analysis revealed that high levels of soluble GroEl and GroES proteins were made
in the induced cells. These results demonstrated that the chaperone hyper-expression
20 system was functional.

EXAMPLE 33

Growth Of BotA/pACYCGro Cell Lines In Fermentation Cultures

Induction of BL21(DE3) cells lacking the LysS plasmid which contained BotA
expression constructs grown in shaker flask or fermentation culture resulted in the
25 expression of primarily insoluble BotA protein. Fermentation cultures were performed
to determine if the simultaneous overexpression of the Gro operon and recombinant *C.*
botulinum type A proteins (BotA proteins) resulted in enhanced solubility of the

recombinant BotA protein. This example involved the fermentation of pHisBotA(syn)kan lacIq T7lac/pACYCGro BL21(DE3) and pHisBotA(syn)kan lacIq T7/pACYCGro BL21(DE3) cell lines. The fermentations were repeated exactly as described in Example 31. Chloramphenicol (34 µg/ml) was included in the feeder and fermentation cultures.

a) **Fermentation Of pHisBotA(syn)kan lacIq
T7lac/pACYCGro BL21(DE3) Cells**

For fermentation of cells containing plasmids comprising the T7lac promoter, induction was with 2 gms IPTG at 1 hr post initiation of glucose feed. The OD₆₀₀ was 35 at time of induction, then 48.5, 61.5, 67 at 1-3 hrs post induction. Viable colony counts decreased from 0-3 hr induction [21 (13), 0, 0, 0; dilution 3 utilized 3 µl of dilution 2 cells] with numbers in parenthesis for the indicating microcolonies. Of 28 colonies scored at the time of induction, 23 retained the pHisBotA(syn)kan lacIq T7lac plasmid (kan resistant), 22 contained the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected). These results were indicative of very strong promoter induction, since colony viability dropped immediately after induction.

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and stained with Coomassie. High level induction of Gro chaperones was observed, but very low level expression of soluble BotA protein was observed, increasing from 1 to 4.0 hrs post induction (no expression detected in uninduced cells). The dramatically lower expression of the BotA antigen in the presence of chaperone may be due to promoter occlusion (*i.e.*, the stronger T7 promoter on the chaperone plasmid is preferentially utilized).

**b) Fermentation Of pHisBotA(syn)kan lacIq T7/
pACYCGro BL21(DE3) Cells**

A fermentation utilizing the T7-driven BotA expression plasmid was performed. Induction was with 1 gm IPTG at 2 hrs post initiation of glucose feed. The OD₆₀₀ was
5 41 at time of induction, then 51.5, 61.5, 61.5 and 66 at 1-4 hrs post induction. Viable
colony counts decreased from 0-4 hrs induction [71, 1 (34), 1 (1), 1, 0; dilution 3
utilized 6 µl dilution 2 cells) with numbers in parenthesis for the uninduced timepoint
indicating microcolonies. Of 65 colonies scored at the time of induction, all 65
retained both the pHisBotA(syn)kan lacIq T7 plasmid (kan resistant) and the chaperone
10 plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan
plates (no mutations detected).

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and
stained with Coomassie. High level induction of Gro chaperones and moderate level
expression of soluble BotA protein was observed, increasing from 1 to 4.0 hrs post
15 induction (no expression detected in uninduced cells).

A PEI-clarified lysate (0.2% final concentration PEI) [850 ml from 130 gm cell
pellet (2 liters fermentation harvest)] was purified on a large scale IDA column. A
total of 78 mg of protein was eluted. Extracts from the purification were resolved on
a 12.5% SDS-PAGE gel and stained with Coomassie. The elution was found to
20 contain an approximately 1:1 mix of BotA/chaperone protein (Figure 32). PEI lysates
prepared in this manner were typically 16 OD₂₈₀/ml. This was estimated to be 8 mg
protein/ml of lysate (by BCA assay). Thus, the eluted recombinant BotA protein
represented 0.55% of the total soluble cellular protein applied to the column.

In Figure 32, lane 1 contains molecular weight markers, lanes 2-9 contain
25 extracts from pHisBotA(syn)kan lacIq T7/pACYCGro/BL21(DE3) cells before or
during purification on the IDA column. Lane 2 contains total protein extract; lane 3
contains soluble protein extract; lanes 4 and 5 contain PEI-clarified lysates
(duplicates); lanes 6 and 7 contain flow-through from the IDA column (duplicates) and
lanes 8 and 9 contain IDA column elute (lane 9 contains 1/10 the amount applied to
30 lane 8).

These results demonstrate, that although the majority of the BotA protein produced was insoluble, 20 mg/liter of soluble recombinant BotA protein can be purified utilizing the pHisBotA(syn)kan lacIq T7/pACYCGro/BL21(DE3) expression system.

5

EXAMPLE 34

Purification Of Recombinant BotA Protein From Folding Chaperones

In this example of size exclusion chromatography was used to purify the recombinant BotA protein away from the folding chaperones and imidazole present in the IDA-purified material (Ex. 33).

10

To enhance the solubility of the recombinant BotA protein during scale-up, the protein was co-expressed with folding chaperones (Ex. 33). As observed with the recombinant BotB protein (Example 40 below), the folding chaperones co-eluted with the recombinant BotA protein during the Ni-IDA purification step. Because the recombinant BotA and BotB proteins have similar molecular weights (about 1/10 the size of the non-reduced folding chaperone) and the imidazole step gradient strategy was unsuccessful in purifying BotB away from the folding chaperone (see Ex. 40), size exclusion chromatography was examined for the ability to purify the recombinant BotA protein away from the folding chaperones.

15

A column (2.5 x 24 cm) containing Sephacryl S-100 HR (Pharmacia) was poured (bed volume ~ 110 ml). Proteins having molecular weights greater than 100 K are expected to elute in the void volume under these conditions and smaller proteins should be retained by the beads and elute at different times, depending on their molecular weights. To maintain solubility of the purified BotA protein, the Sephacryl column was equilibrated in a buffer having the same salt concentration as the buffer used to elute the BotA protein from the IDA column (*i.e.*, 50 mM sodium phosphate, 0.5 M NaCl, 10% glycerol; all reagents from Mallinkrodt, Chesterfield, MO).

20

25

Five milliliters of the IDA-purified recombinant BotA protein (Ex. 33) was filtered through a 0.45 μ syringe filter, applied to the column and the equilibration

buffer was pumped through the column at a flow rate of 1 ml/minute. Eluted proteins were monitored by absorbance at 280 nm and collected either manually or with a fraction collector (BioRad). Appropriate fractions were pooled, if necessary, and the protein was quantitated by absorbance at 280 nm and/or BCA protein assay (Pierce).

5 The isolated peaks were then analyzed by native and/or SDS-PAGE to identify the proteins present and to evaluate purity. The folding chaperone eluted first, followed by the recombinant BotA protein and then the imidazole peak.

SDS-PAGE analysis (12.5% polyacrylamide, reduced samples) was used to evaluate the purity of the IDA-purified recombinant BotA protein before and after
10 S-100 purification. Figure 33 shows the difference in purity before and after the S-100 purification step. In Figure 33, lane 1 contains molecular weight markers (BioRad broad range). Lane 2 shows the IDA-purified recombinant BotA protein preparation, which is contaminated with significant amounts of the folding chaperone. Following S-100 purification, the amount of folding chaperone present in the BotA
15 sample is reduced dramatically (lane 3). Lane 4 contains no protein (*i.e.*, it is a blank lane); lanes 5-8 contain samples of IDA-purified recombinant BotB and BotE proteins and are discussed *infra*.

Endotoxin levels in the S-100 purified BotA preparation were determined using the LAL assay (Associates of Cape Cod) as describe in Example 24. The purified
20 BotA preparation was found to contain 22.7 to 45.5 EU/mg recombinant protein.

These results demonstrate that size exclusion chromatography was successful in purifying the recombinant BotA protein from folding chaperones and imidazole following an initial IDA purification step. Furthermore, these results demonstrate that the S-100 purified BotA protein was substantially free of endotoxin.

EXAMPLE 35

Cloning And Expression Of The C Fragment Of The *C. botulinum* Serotype B Toxin Gene

The *C. botulinum* type B neurotoxin gene has been cloned and sequenced
5 [Whelan *et al.* (1992) Appl. Environ. Microbiol. 58:2345 and Hutson *et al.* (1994)
Curr. Microbiol. 28:101]. The nucleotide sequence of the toxin gene derived from the
Eklund 17B strain (ATCC 25765) is available from the EMBL/GenBank sequence data
banks under the accession number X71343; the nucleotide sequence of the coding
region is listed in SEQ ID NO:39. The amino acid sequence of the *C. botulinum* type
10 B neurotoxin derived from the strain Eklund 17B is listed in SEQ ID NO:40. The
nucleotide sequence of the *C. botulinum* serotype B toxin gene derived from the
Danish strain is listed in SEQ ID NO:41 and the corresponding amino acid sequence is
listed in SEQ ID NO:42.

The DNA sequence encoding the native *C. botulinum* serotype B C fragment
15 gene derived from the Eklund 17B strain can be expressed using the pETHisb vector;
the resulting coding region is listed in SEQ ID NO:43 and the corresponding amino
acid sequence is listed in SEQ ID NO:44. The DNA sequence encoding the native *C.*
botulinum serotype B C fragment gene derived from the Danish strain can be
expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID
20 NO:45 and the corresponding amino acid sequence is listed in SEQ ID NO:46. The C
fragment region from any strain of *C. botulinum* serotype B can be amplified and
expressed using the approach illustrated below using the C fragment derived from *C.*
botulinum type B 2017 strain.

The *C. botulinum* type B neurotoxin gene is synthesized as a single polypeptide
25 chain which is processed to form a dimer composed of a light and a heavy chain
linked via disulfide bonds; the type B neurotoxin has been reported to exist as a
mixture of predominately single chain with some double chain (Whelan *et al.*, supra).
The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C

fragment or the H_C domain. Expression of the C fragment of *C. botulinum* type B toxin in heterologous hosts (e.g., *E. coli*) has not been previously reported.

The native C fragment of the *C. botulinum* serotype B toxin gene was cloned and expression constructs were made to facilitate protein expression in *E. coli*. This example involved PCR amplification of the gene, cloning, and construction of expression vectors.

The C fragment of the *C. botulinum* serotype B (BotB) toxin gene was cloned using the protocols and conditions described in Example 28 for the isolation of the native BotA gene. The *C. botulinum* type B 2017 strain was obtained from the American Type Culture Collection (ATCC #17843). The following primer pair was used to amplify the BotB gene: 5'-CGCCATGGCTGATACAATACTAATAGAAATG-3' [5' primer, engineered *Nco*I site underlined (SEQ ID NO:47)] and 5'-GCAAGCTTTTATTTCAGTCCACCCTTCATC-3' [3' primer, engineered *Hind*III site underlined, native gene termination codon italicized (SEQ ID NO:48)]. After cloning into the pCRscript vector, the *Nhe*I(filled)/*Hind*III fragment was cloned into pETHisb vector as described for BotA C fragment gene in Example 28. The resulting construct was termed pHisBotB.

pHisBotB expresses the BotB gene sequences under the transcriptional control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag. The pHisBotB expression construct was transformed into BL21(DE3) pLysS competent cells and 1 liter cultures were grown, induced and his-tagged proteins were purified utilizing a NiNTA resin (eluted in low pH elution buffer) as described in Example 28. Total, soluble and purified proteins were resolved by SDS-PAGE and detected by Coomassie staining and Western blot hybridization utilizing a chicken anti-*C. botulinum* serotype B toxoid primary antibody (generated by immunization of hens using *C. botulinum* serotype B toxoid as described in Example 3). Samples of BotA and BotE C fragment proteins were included on the gels for MW and immunogenicity comparisons. Strong immunoreactivity to only the BotB protein was detected with the anti-*C. botulinum* serotype B toxoid antibodies. The

recombinant BotB protein was expressed at low levels (3 mg/liter) as a soluble protein. The purified BotB protein migrated as a single band of the predicted MW (*i.e.*, ~50kD).

These results demonstrate the cloning of the native *C. botulinum* serotype B C fragment gene, the expression and purification of the recombinant BotB protein as a soluble his-tagged protein in *E. coli*.

EXAMPLE 36

Generation Of Neutralizing Antibodies Using The Recombinant pHisBotB Protein

The ability of the purified pHisBot protein to generate neutralizing antibodies was examined. Nine BALBc mice were immunized with BotB protein (purified as described in Ex. 35) using Gerbu GMDP adjuvant (CC Biotech). The low pH elution was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received a subcutaneous injection of 100 μ l antigen/adjuvant mix (15 μ g antigen + 1 μ g adjuvant) on day 0. Mice were subcutaneously boosted as above on day 14 and bled on day 28. Mice were subsequently boosted 1-2 weeks after bleeding and were then bled on day 70.

Anti-*C. botulinum* serotype B toxoid titers were determined in day 28 serum from individual mice from each group using the ELISA protocol outlined in Example 29 with the exception that the plates were coated with *C. botulinum* serotype B toxoid, and the primary antibody was a chicken anti-*C. botulinum* serotype B toxoid.

Seroconversion [relative to control mice immunized with pHisBotE antigen (described below)] was observed with all 9 mice immunized with the purified pHisBotB protein.

The ability of the anti-BotB antibodies to neutralize native *C. botulinum* type B toxin was tested in a mouse-*C. botulinum* neutralization model using pooled mouse serum (see Ex. 23b). The LD₅₀ of purified *C. botulinum* type B toxin complex (Dr. Eric Johnson, University of Wisconsin, Madison) was determined by a intraperitoneal (IP) method [Schantz and Kautler (1978), *supra*] using 18-22 g female ICR mice. The amount of neutralizing antibodies present in the serum of the immunized mice was

determined using serum antibody titrations. The various serum dilutions (0.01 ml) were mixed with 5 LD₅₀ units of *C. botulinum* type B toxin and the mixtures were injected IP into mice. The neutralizations were performed in duplicate. The mice were then observed for signs of botulism for 4 days. Undiluted serum (day 28 or day 70) was found to protect 100% of the injected mice while the 1:10 diluted serum did not. This corresponds to a neutralization titer of 0.05-0.5 IU/ml.

These results demonstrate that seroconversion occurred and neutralizing antibodies were induced when the pHisBotB protein was utilized as the immunogen.

EXAMPLE 37

Construction Of Vectors To Facilitate Expression Of His-Tagged BotB Protein In Fermentation Cultures

A number of expression vectors were constructed to facilitate the expression of recombinant BotB protein in large scale fermentation culture. These constructs varied as to the strength of the promoter utilized (T7 or T7lac) and the presence of repressor elements (lacIq) on the plasmid. The resulting constructs varied in the level of expression achieved and in plasmid stability which facilitated the selection of a optimal expression system for fermentation scaleup.

The BotB expression vectors created for fermentation culture were engineered to utilize the kanamycin rather than the ampicillin resistance gene, and contained either the T7 or T7lac promoter, with or without the lacIq gene for the reasons outlined in Example 30.

In all cases, the protein expressed by the various expression vectors is the pHisBot B protein described in Example 35, with the only differences between clones being the alteration of various regulatory elements. Using the designations outlined below, the pHisBotB clone (Ex. 35) is equivalent to pHisBotB amp T7lac.

a) Construction Of pHisBotB kan T7lac

pHisBotB kan T7lac was constructed by insertion of the *BglII/HindIII* fragment of pHisBotB which contains the BotB gene sequences into the pPA1870-2680 kan T7lac vector which had been digested with *BglII* and *HindIII* (the pPA1870-2680 kan T7lac vector contains the pET24 kan gene in the pET23 vector, such that no lacIq gene is present). Proper construction of pHisBotB kan T7lac was confirmed by restriction digestion.

b) Construction Of pHisBotB kan lacIq T7lac

pHisBotB kan lacIq T7lac was constructed by insertion of the *BglII/HindIII* fragment of pHisBotB which contains the BotB gene sequences into similarly cut pET24a vector. Proper construction of pHisBotB kan lacIq T7lac was confirmed by restriction digestion.

c) Construction Of pHisBotB kan lacIq T7

pHisBotB kan lacIq T7 was constructed by inserting the *NdeI/XhoI* fragment from pHisBotE kan lacIq T7lac which contains the BotB gene sequences into similarly cleaved pPA1870-2680 kan lacIq T7 vector (this vector contains the T7 promoter, the same N-terminal his-tag as the Bot constructs, the *C. difficile* toxin A insert, and the kan lacIq genes; this cloning replaces the *C. difficile* toxin A insert with the BotB insert). Proper construction was confirmed by restriction digestion.

Expression of recombinant BotB protein from these expression vectors and purification of the BotB protein is described in Example 38 below.

EXAMPLE 38

Fermentation And Purification Of Recombinant BotB Protein Utilizing The pHisBotB kan lacIq T7lac, pHisBotB kan T7lac And pHisBotB kan lacIq T7 Vectors

5 The pHisBotB kan lacIq T7lac, pHisBotB kan T7lac and BotB kan lacIq T7 constructs [all transformed into the BI21(DE3) strain] were grown in fermentation cultures to determine the utility of the various constructs for large scale expression and purification of soluble BotB protein. All fermentations were performed as described in Example 31.

a) Fermentation Of pHisBotB kan lacIq T7lac/BI21(DE3)

10 Cells

The fermentation culture was induced 45 min post start of glucose feed with 1 gm IPTG (final concentration = 0.4 mM). pH was maintained at 6.5 rather than 7.0. The OD₆₀₀ was 27 at time of induction, then 35, 38, and 40 at 1-3 hrs post induction. Duplicate platings of diluted 1 hr induction samples (dilutions were prepared as described Ex. 31, dilution 3 utilized 3 µl of dilution 2 cells) on TSA and LB+kan plates yielded 89 TSA colonies and 81 kan colonies (90% kan resistant).

15 Total and soluble protein extracts were resolved on a 12.5% SDS-PAGE gel and total protein was detected by staining with Coomassie blue. Low level induction of insoluble Bot B protein was observed, increasing from 1 to 3 hrs post induction (no expression was detected in uninduced cells).

b) Fermentation Of pHisBotB kan T7lac/BI21(DE3) Cells

25 The fermentation culture was induced 1 hr post start of glucose feed with 2 gm IPTG (final concentration = 0.8 mM). pH was maintained at 6.5 rather than 7.0. The OD₆₀₀ was 24.5 at time of induction, then 31.5, 32, and 33 at 1-3 hrs post induction, respectively. Duplicate platings of diluted 0 hr and 2 hr induction samples (dilutions were prepared as described Ex. 31; dilution 3 utilized 3 µl of dilution 2 cells) on TSA and LB+kan plates yielded 32 TSA colonies and 54 kan colonies (all kan resistant) for

uninduced cells, and 1 TSA colony and 0 kan colonies 2 hr post induction. These results were indicative of strong induction, since viable counts decreased dramatically 2 hrs post induction.

5 Total and soluble extracts were resolved on a 10% SDS-PAGE gel and total protein was detected by staining with Coomassie blue. Moderate induction of insoluble BotB protein was observed, increasing from 1 to 3 hrs post induction (no expression was detected in uninduced cells).

**c) Fermentation Of pHisBotB kan lacIq T7/BI21(DE3)
Cells**

10 The fermentation was induced 2 hr post start of glucose feed with 4 gm IPTG (final concentration = 1.6 mM). pH was maintained at 6.5 rather than 7.0. The OD₆₀₀ was 45 at time of induction, then 47, 50, and 50 and 55 at 1-4 hrs post induction, respectively. Viable colony counts decreased after induction (96, 1, 1, 2, 3; dilution 3 utilized 3 µl of dilution 2 cells). Of 63 colonies scored at the time of induction, all 63
15 retaining the BotB plasmid (kan resistant) and no colonies at induction grew on IPTG + Kan plates (no mutations detected).

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and total protein was detected by staining with Coomassie blue. Moderate level induction of insoluble BotB protein was observed, increasing from 1 to 4 hrs post induction (lower
20 level expression was detected in uninduced cells, since the T7 rather than T7lac promoter was utilized).

**d) Purification Of pHisBotB Protein From pHisBotB
amp T7lac/BI21(DE3) Cells**

25 Soluble recombinant BotB protein was purified utilizing NiNTA resin from 80 ml of cell lysate generated from cells harvested from a pHisBotB fermentation [using the pHisBotB amp T7lac/BI21(DE3) strain]. As predicted from the small scale results above, the majority of the induced protein was insoluble. As well, the eluted material was contaminated with multiple *E. coli* contaminant proteins. A Coomassie blue-

stained SDS-PAGE gel containing extracts derived from pHisBotB amp T7lac/BI21(DE3) cells before and during purification is shown in Figure 34. In Figure 34, lane 1 contains broad range protein MW markers (BioRad). Lanes 2-5 contain extracts prepared from pHisBotB amp T7lac/BI21(DE3) cells grown in fermentation culture; lane 2 contains total protein; lane 3 contains soluble protein; lane 4 contains protein which did not bind to the NiNTA column (*i.e.*, the flow-through) and lane 5 contains protein eluted from the NiNTA column.

Similar results were obtained using a small scale IDA column utilizing a cell lysate from the pHisBotB kan lacIq T7 fermentation described above. 250 mls of a 20% w/v PEI clarified lysate (50 gms cell pellet) of botB kan lacIq T7/BI21(DE3) cells were purified on a small scale IDA column. The total yield of eluted protein was 21 mg protein (assuming 1 mg/ml solution = 2 OD₂₈₀/ml). When analyzed by SDS-PAGE and Coomassie staining, the BotB protein was found to comprise approximately 50% of the eluted protein with the remainder being a ladder of *E. coli* proteins similar to that observed with the NiNTA purification.

The NiNTA alkaline phosphatase conjugate was utilized to detect his-tagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. The results demonstrated that a small percentage of BotB protein was soluble, that the soluble protein was not precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 1 liter fermentation harvest yielded a 67.5 gm cell pellet, this indicated that the yield of soluble affinity purified BotB protein from the IDA column was 14 mg/liter.

EXAMPLE 39

Co-Expression Of Recombinant BotB Proteins And Folding Chaperones In Fermentation Cultures

5 Fermentations were performed to determine if the simultaneous overexpression
of folding chaperones (*i.e.*, the Gro operon) and the BotB protein resulted in enhanced
solubility of the Bot B protein. This example involved fermentation of the
pHisBotBkan lacIq T7lac/pACYCGro BL21(DE3), pHisBotB kan T7lac/pACYCGro
BI21(DE3) and pHisBotBkan lacIq T7/ pACYCGro BL21(DE3) cell lines.
10 Fermentation was carried out as described in Example 31; 34 µg/ml chloramphenicol
was included in the feeder and fermentation cultures.

a) Fermentation Of pHisBotBkan lacIq T7lac/pACYCGro BL21(DE3) Cells

Induction was with 4 gms IPTG at 1 hr 15 min post initiation of the glucose
feed. The OD₆₀₀ was 38 at time of induction, then 50, 58.5, 62 and 68 at 1-4 hrs post
15 induction. Viable colony counts decreased during induction (24, 0, 0, 2, 0 at 0-4 hr
induction; dilution 3 utilized 3 µl of dilution 2 cells). Of 24 colonies scored at the
time of induction, 24 retained the BotB plasmid (kan resistant), 24 contained the
chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on
IPTG+Kan plates (no mutations detected).

20 Total and soluble extracts were resolved on 12.5% SDS-PAGE gels and were
either stained with Coomassie blue or subjected to Western blotting (his-tagged
proteins were detected utilizing the NiNTA-alkaline phosphatase conjugate). This
analysis revealed that the Gro chaperones were induced to high levels, but very low
level expression of soluble BotB protein was observed, increasing from 1 to 4.0 hrs
25 post induction (no expression detected in uninduced cells, induced protein detected
only on Western blot). The dramatically lower expression of BotB protein in the
presence of chaperone may be due to promoter occlusion (*i.e.*, the stronger T7
promoter on the chaperone plasmid was preferentially utilized).

**b) Fermentation Of pHisBotB kan
T7lac/pACYCGro/BI21(DE3) Cells**

Induction was with 4 gms IPTG at 1 hr post initiation of the glucose feed. The OD₆₀₀ was 33.5 at time of induction, then 44, 51, 58.5 and 69 at 1-4 hrs post induction. Viable colony counts decreased after 2 hrs induction (43, 65, 74, 0 (70), 0 (70) at 0-4 hr induction; bracketed numbers represent microcolonies; dilution 3 utilized 3 µl of dilution 2 cells). Most colonies at induction retained the BotB plasmid (kan resistant) and the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected).

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and subjected to Western blotting; his-tagged proteins were detected utilizing the NiNTA-alkaline phosphatase conjugate. This analysis revealed that the Gro chaperones were induced to high levels and low level expression of soluble Bot B protein was observed, increasing from 1 to 4.0 hrs post induction (no expression detected in uninduced cells).

A small scale IDA purification of BotB protein from a 250 ml PEI clarified 15% w/v extract (37.5 gm cell pellet) yielded approximately 12.5 mg protein, of which approximately 50% was BotB protein and 50% was GroEL chaperone (assessed by Coomassie staining of a 10% SDS-PAGE gel). The NiNTA alkaline phosphatase conjugate was utilized to detect his-tagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. The results demonstrated that all of the BotB protein produced by the pHisBotB kan T7lac/pACYCGro/BI21(DE3) cells was soluble; the BotB protein was not precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 1 liter fermentation harvest yielded a 75 gm cell pellet, this indicated that the yield of soluble affinity purified bot B protein from this fermentation was 12.5 mg/liter. These results also demonstrated that additional purification steps are necessary to separate the chaperone proteins from the BotB protein.

c) **Fermentation Of pHisBotBkan lacIq**

T7/pACYCGro/BL21(DE3) Cells

Induction was with 4 gms IPTG at 2 hr post initiation of the glucose feed. The OD₆₀₀ was 46 at time of induction, then 56, 63, 69 and 71.5 at 1-4 hrs post induction. Viable colony counts decreased after induction (58, 3(5), 3, 0, 0 at 0-4 hr induction; bracketed numbers represent microcolonies; dilution 3 utilized 3 µl of dilution 2 cells). All (53/53) colonies scored at the time of induction retained the BotB plasmid (kan resistant) and the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected).

Total and soluble extracts were resolved on a 10% SDS-PAGE gels and Western blotted and his-tagged proteins were detected utilizing the NiNTA-alkaline phosphatase conjugate. This analysis revealed that the Gro chaperones were induced to high levels (observed by ponceau S staining), and a much higher expression of soluble Bot B protein (compared to expression in the pHisBotB kan T7lac/pACYCGro fermentation) was observed at all timepoints, including uninduced cells (some increase in BotB protein levels were observed after induction).

A small scale IDA purification of BotB protein from a 100 ml PEI clarified 15% w/v extract (15 gm cell pellet) yielded approximately 40 mg protein, of which approximately 50% was BotB protein and 50% was GroEL chaperone, as assessed by Coomassie staining of a 10% SDS-PAGE gel. The NiNTA alkaline phosphatase conjugate was utilized to detect his-tagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. The results demonstrated that a significant percentage (i.e., ~10-20 %) of BotB protein was soluble, that the solubilized protein was not precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 10 liter fermentation yielded a 108 gm cell pellet, this indicated that the yield of soluble affinity purified BotB protein from this fermentation was 144 mg/liter.

In a scale up experiment, 2 liters of a 20% w/v PEI clarified lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells were purified on a large scale IDA column. The purification was performed in duplicate. The total yield of BotB

protein was 220 and 325 mgs protein in the two experiments (assuming 1 mg/ml solution = 2.0 OD₂₈₀/ml). This represents 0.7% or 1.0%, respectively, of the total soluble cellular protein (assuming a PEI lystate having a concentration of 8 mg protein/ml and that the eluted material comprises a 1:1 mixture of BotB and folding chaperone). The NiNTA alkaline phosphatase conjugate was utilized to detect his-tagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. These results demonstrated that a significant percentage (*i.e.*, ~10-20 %) of the BotB protein was soluble, that the solubilized protein was not precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 1 liter fermentation harvest yielded a 108 gm cell pellet, this indicated that the yield of soluble affinity purified BotB protein from the large scale purification was 60 mg or 89 mg/liter. These results also demonstrated that further purification would be necessary to remove the contaminating chaperone protein.

The above results provide methodologies for the purification of soluble BotB protein from fermentation cultures, in a form contaminated predominantly with a single *E. coli* protein (the folding chaperone utilized to enhance solubility). In the next example, methods are provided for the removal of the contaminating chaperone protein.

EXAMPLE 40

Removal Of Contaminating Folding Chaperone Protein From Purified Recombinant *C. botulinum* Type B Protein

In this example size exclusion chromatography and ultrafiltration was used to purify recombinant BotB protein from the folding chaperones and imidazole in IDA-purified material.

To enhance the solubility of the recombinant BotB protein during scale-up, the protein was co-expressed with folding chaperones (see Ex. 39). During the Ni-IDA purification step, the folding chaperones co-eluted with the BotB protein in 800 mM

imidazole; therefore, a second purification step was required to isolate the BotB free of folding chaperones. Lane 3 of Figure 35 contains proteins eluted from an IDA column to which a lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells had been applied; the proteins were resolved on a 4-15% polyacrylamide pre-cast gradient gel (Bio-Rad, Hercules, CA) run under native conditions and then stained with Coomassie blue. In Figure 35, lanes 1 and 4 contain proteins present in peak 1 and peak 2 from a Sephacryl S-100 column run as described below; lane 2 is blank.

As seen in lane 3 of Figure 35, the IDA-purified sample consists primarily of the folding chaperones and the BotB protein. The fact that the chaperones and the BotB antigen appear as two distinct bands under native conditions suggested they were not complexed together and therefore, it should be possible to separate them, using either a gradient of imidazole concentrations or size exclusion methods.

In order to determine whether a gradient of imidazole concentrations could be used to separate the chaperone from the BotB protein, a step gradient using imidazole at 200, 400, 600, and 800 mM in 50 mM sodium phosphate, 0.5 M NaCl and 10 % glycerol, pH 6.8 was applied to an IDA column (containing proteins bound from a lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells). By narrowing the range of imidazole concentrations, it was hoped that the BotB and chaperone proteins would differentially elute at different concentrations of imidazole. Eluted proteins were monitored by absorbance at 280 nm and collected either manually or with a fraction collector (BioRad). Protein was found to elute at 200 and 400 mM imidazole only.

Figure 36 shows a Coomassie stained SDS-PAGE gel containing protein eluted during the imidazole step gradient. Lane 1 contains broad range MW markers (BioRad). Lane 2 contains BotB protein purified by IDA chromatography of an extract of pHisBotB/BL21(DE3) pLysS cells grown in shaker flask culture (*i.e.*, no co-expression of chaperones; Ex. 35). Lane 3 contains a 20% w/v PEI clarified lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells (*i.e.*, the lysate prior to purification by IDA chromatography). Lanes 4 and 5 contain protein which eluted at

200 or 400 mM imidazole, respectively. Lane 6 is blank. Lanes 7 and 8 contain 1/5 the load present in lanes 4 and 5.

As shown in Figure 36, both the chaperone and the BotB protein eluted in 200 mM imidazole, and more chaperone elutes in 400 mM imidazole, however no concentration of imidazole tested permitted the elution of BotB protein alone. Consequently, no significant purification was achieved using imidazole at these concentrations.

Because of the considerable difference in molecular weights between the folding chaperone, which is a multimer with a total molecular weight around 400 kD (as determined on a Shodex KB 804 sizing column by HPLC), and the recombinant BotB protein (molecular weight around 50 kD), size exclusion chromatography was next examined for the ability to separate these proteins.

a) Size Exclusion Chromatography

A column containing Sephacryl S-100 HR (S-100) (Pharmacia) was poured (2.5 cm x 24 cm ; ~110 ml bed volume). The column was equilibrated in a buffer consisting of phosphate buffered saline (10mM potassium phosphate, 150 mM NaCl, pH 7.2) and 10 % glycerol (Mallinkrodt). Typically, 5 ml of the IDA-purified BotB protein was filtered through a 0.45 μ syringe filter and applied to the column, and the equilibration buffer was pumped through the column at a flow rate of 1 ml/minute. Eluted proteins were monitored by absorbance at 280 nm and collected either manually or with a fraction collector. Appropriate tubes were pooled, if necessary, and the protein was quantitated by absorbance at 280 nm and/or by BCA protein assay. The isolated peaks were then analyzed by native and/or SDS-PAGE to identify the protein and evaluate the purity.

Because of its larger size, the folding chaperone eluted first, followed by the recombinant BotB protein. A smaller third peak was observed which failed to stain when analyzed by SDS-PAGE and therefore was presumed to be imidazole.

SDS-PAGE analysis (12.5% polyacrylamide, reduced samples) was used to evaluate the purity of the IDA-purified recombinant BotB protein before and after S-100 purification. The results are shown in Figure 33.

In Figure 33, lane 1 contains broad range MW markers (BioRad). Lane 5 contains IDA-purified BotB protein. Lane 6 contains IDA-purified BotB protein following S-100 purification. Lane 7 is blank (lanes 2-4 were discussed in Ex. 34 above).

The results shown in Figure 33 show that the IDA-purified BotB is significantly contaminated with the folding chaperone (molecular weight about 60 kD under reducing conditions; lane 6). Following S-100 purification, the amount of folding chaperone present in the BotB sample was reduced dramatically (lane 7). Visual inspection of the Coomassie stained SDS-PAGE gel revealed that after S-100 purification, > 90% of the total protein present was BotB.

The IDA-purified BotB and the S-100-purified BotB samples were analyzed by HPLC on a size exclusion column (Shodex KB 804); this analysis revealed that the BotB protein represented 64% of the total protein in the IDA-purified sample and that following S-100 purification, the BotB protein represented >95% of the total protein in the sample.

The IDA-purified BotB material was also applied to a ACA 44 (SpectraPor, Houston, TX) column. The ACA 44 resin is equivalent to the S-100 resin and chromatography using the ACA 44 resin was carried out exactly as described above for the S-100 resin. The ACA 44 resin was found to separate the recombinant BotB protein from the folding chaperone. The ACA 44-purified BotB sample was analyzed for endotoxin using the LAL assay (Associates of Cape Cod) as describe in Example 24. Two aliquots of the ACA 44-purified BotB preparation were analyzed and were found to contain either 58 to 116 EU/mg recombinant protein or 94 to 189 EU/mg recombinant protein.

These results demonstrate that size exclusion chromatography can be used to purify the recombinant BotB protein from the folding chaperone and imidazole in IDA-purified material.

b) Ultrafiltration For The Separation Of Recombinant BotB Protein And Chaperones

Ultrafiltration was examined as an alternative method for the separation recombinant BotB protein and folding chaperones in IDA-purified material. While in this example only mixtures of BotB and chaperones were separated by ultrafiltration, this technique is suitable for use with recombinant BotA and BotE proteins as well provided that the wash buffers used are altered as necessary to take into account different requirements for solubility.

The recombinant BotB protein and folding chaperones were separated using a two-step sequential ultrafiltration method. The first membrane used had a nominal molecular weight cutoff (MWCO) of approximately 100 kD; this membrane retains the larger folding chaperone while allowing the smaller recombinant protein to pass through. The addition of several volumes of wash buffer may be required to efficiently wash the recombinant protein through the membrane. The second step utilized a membrane with a nominal MWCO of approximately 10 kD. During this step, the recombinant antigen was retained by the membrane and could be concentrated to the degree desired and the imidazole and excess wash buffer passed through the membrane.

Twenty-seven milliliters of an IDA-purified BotB preparation was ultrafiltered through a 47 mm YM 100 (100 kD MWCO) membrane (Amicon) in a 50 ml stirred cell (Amicon). The membrane was washed in dd H₂O prior to use as recommended by the manufacturer. Six volumes of 10% glycerol in PBS were washed through to remove most of the recombinant BotB protein and this wash was collected in a separate vessel. The resulting BotB protein-rich filtrate was then concentrated 12-fold using a YM 10 (10 kD MWCO) membrane (Amicon), to a final volume of 14 ml. The YM 100 and YM 10 concentrates were analyzed along with the lysate starting material by native PAGE using a 4 -15% pre-cast gradient gel (BioRad). The results are shown in Figure 37.

In Figure 37, lane 1 contains IDA-purified BotB derived from a shaker flask culture (*i.e.*, no co-expression of chaperones; Ex. 35); lane 2 contains a 20% w/v PEI

clarified lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells; lane 3 shows the lysate of lane 3 after IDA purification; lane 4 contains the YM 10 concentrate and lane 5 contains the YM 100 concentrate.

5 The results shown in Figure 37 demonstrate that the recombinant BotB protein can be purified away from the folding chaperone by ultrafiltration through a 100 kD MWCO membrane (lane 4), leaving the chaperone protein in the 100 kD concentrate (lane 5). Analysis of the sample in lane 5 also showed that very little of the BotB protein was retained by the 100 kD MWCO membrane after 6 volumes of wash buffer had been applied.

10 The BotB samples following IDA chromatography and following ultrafiltration through the YM 100 membrane were analyzed by HPLC on a size exclusion column (Shodex KB 804); this analysis revealed that the BotB protein represented 64% of the total protein in the IDA-purified sample and that following ultrafiltration through the YM 100 membrane, the BotB protein represented >96% of the total protein in the
15 sample.

The BotB protein purified by ultrafiltration through the YM 100 membrane was examined for endotoxin using the LAL assay (Associates of Cape Cod) as describe in Example 24. Two aliquots of the YM 100-purified BotB preparation were analyzed and were found to contain either 18 to 36 EU/mg recombinant protein or 125 to 250
20 EU/mg recombinant protein.

The above results demonstrate that size exclusion chromatography and ultrafiltration can be used to purify recombinant botulinal toxin proteins away from folding chaperones.

EXAMPLE 41

Cloning And Expression Of The C Fragment Of The *C. botulinum* Serotype E Toxin Gene

The *C. botulinum* type E neurotoxin gene has been cloned and sequenced from
5 several different strains [Poulet *et al.* (1992) Biochem. Biophys. Res. Commun.
183:107 (strain Beluga); Whelan *et al.* (1992) Eur. J. Biochem. 204:657 (strain NCTC
11219); Fujii *et al.* (1990) Microbiol. Immunol. 34:1041 (partial sequence of strains
Mashike, Iwani and Otaru) and Fujii *et al.* (1993) J. Gen. Microbiol. 139:79 (strain
Mashike)]. The nucleotide sequence of the type E toxin gene is available from the
10 EMBL sequence data bank under accession numbers X62089 (strain Beluga) and
X62683 (strain NCTC 11219). The nucleotide sequence of the coding region (strain
Beluga) is listed in SEQ ID NO:49. The amino acid sequence of the *C. botulinum*
type E neurotoxin derived from strain Beluga is listed in SEQ ID NO:50. The
nucleotide sequence of the coding region (strain NCTC 11219) is listed in SEQ ID
15 NO:51. The amino acid sequence of the *C. botulinum* type E neurotoxin derived from
strain NCTC 11219 is listed in SEQ ID NO:52.

The DNA sequence encoding the native *C. botulinum* serotype E C fragment
gene derived from the Beluga strain can be expressed as a histidine-tagged protein
using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:53 and
20 the corresponding amino acid sequence is listed in SEQ ID NO:54. The DNA
sequence encoding the C fragment of the native *C. botulinum* serotype E gene derived
from the NCTC 11219 strain can be expressed as a histidine-tagged fusion protein
using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:55 and
the corresponding amino acid sequence is listed in SEQ ID NO:56. The C fragment
25 region from any strain of *C. botulinum* serotype E can be amplified and expressed
using the approach illustrated below using the C fragment derived from *C. botulinum*
type E 2231 strain (ATCC #17786).

The type E neurotoxin gene is synthesized as a single polypeptide chain which may be converted to a double-chain form (*i.e.*, a heavy chain and a light chain) by cleavage with trypsin; unlike the type A neurotoxin, the type E neurotoxin exists essentially only in the single-chain form. The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H_C domain. Expression of the C fragment of *C. botulinum* type E toxin in heterologous hosts (*e.g.*, *E. coli*) has not been previously reported.

The native C fragment of the *C. botulinum* serotype E toxin (BotE) gene was cloned and inserted into expression vectors to facilitate expression of the recombinant BotE protein in *E. coli*. This example involved PCR amplification of the gene, cloning, and construction of expression vectors.

The BotE serotype gene was isolated using PCR as described for the BotA serotype gene in Example 28. The *C. botulinum* type E strain was obtained from the American Type Culture Collection (ATCC #17786; strain 2231). The following primer pair was used in the PCR amplification: 5'-CGCCATGGCTCTTTCTTCTTATACAGATGAT-3' (5' primer, engineered *Nco*I site underlined) (SEQ ID NO:57) and 5'-GCAAGCTTTTATTTTCTTGCCATCCATG-3' (3' primer, engineered *Hind*III site underlined, native gene termination codon italicized) (SEQ ID NO:58). The PCR product was inserted into pCRscript as described in Example 28. The resulting pCRscript BotE clone was confirmed by restriction digestion, as well as, by obtaining the sequence of approximately 300 bases located at the 5' end of the C fragment coding region using standard DNA sequencing methods. The resulting BotE sequence was identical to that of the published *C. botulinum* type E toxin sequence [Whelan *et al* (1992), *supra*].

The *Nhe*I(filled)/*Hind*III fragment from a pCRscript BotE recombinant was cloned into pETHisb vector as described for BotA C fragment in Example 28. The resulting construct was termed pHisBotE. pHisBotE expresses the BotE gene under the control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag.

The pHisBotE expression construct was transformed into BL21(DE3) pLysS competent cells and 1 liter cultures were grown, induced and his-tagged proteins were purified utilizing a NiNTA resin (eluted in low pH elution buffer) as described in Example 28. Total, soluble and purified proteins were resolved by SDS-PAGE and detected by Coomassie staining. The results are shown in Figure 38.

In Figure 38, lane 1 contains broad range MW markers (BioRad); lane 2 contains a total protein extract; lane 3 contains a soluble protein extract; lane 4 contains proteins present in the flow through from the NiNTA column (this sample was not diluted prior to loading and therefore represents a load 5X that of the load applied for the total and soluble extracts in lanes 2 and 3); lane 5 contains proteins eluted from the NiNTA column; lane 6 contains protein eluted from a NiNTA column which had been stored at -20°C for 1 year.

The pHisBotE protein was expressed at moderate levels (7 mg/liter) as a totally soluble protein. The purified protein migrated as a single band of the predicted MW.

Western blot hybridization utilizing a chicken anti-*C. botulinum* serotype E toxoid primary antibody (generated by immunization of hens as described in Example 3 using *C. botulinum* serotype E toxoid) was also performed on the total, soluble and purified BotE proteins. Samples of BotA and BotB C fragments were also included on the gels to facilitate MW and immunogenicity comparisons. Strong immunoreactivity was detected using the anti-*C. botulinum* type E toxoid antibody only with the BotE protein.

These results demonstrate that the native BotE gene sequences can be expressed as a soluble his-tagged protein in *E. coli* and purified by metal-chelation affinity chromatography.

EXAMPLE 42

Generation Of Neutralizing Antibodies Using The Recombinant pHisBotE Protein

The ability of the purified pHisBotE protein to generate neutralizing antibodies was examined. Nine BALBc mice were immunized with BotE protein (purified as described in Ex. 41) using Gerbu GMDP adjuvant (CC Biotech). The low pH elution was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received a subcutaneous injection of 100 μ l antigen/adjuvant mix (35 μ g antigen + 1 μ g adjuvant) on day 0. Mice were subcutaneously boosted as above on day 14 and bled on day 28. Mice were subsequently boosted and bled on day 70.

Anti-*C. botulinum* serotype E toxoid titers were determined in day 28 serum from individual mice from each group using the ELISA protocol outlined in Example 29 with the exception that the plates were coated with *C. botulinum* serotype E toxoid, and the primary antibody was a chicken anti-*C. botulinum* serotype E toxoid. Seroconversion [relative to control mice immunized with the p6xHisBotA antigen (Ex. 29)] was observed with all 9 mice immunized with the purified pHisBotE protein.

The ability of the anti-BotE antibodies to neutralize native *C. botulinum* type E toxin was tested in a mouse-*C. botulinum* neutralization model using pooled mouse serum (see Ex. 23b). The LD₅₀ of purified *C. botulinum* type E toxin complex (Dr. Eric Johnson, University of Wisconsin, Madison) was determined by a intraperitoneal (IP) method [Schantz and Kautler (1978), supra] using 18-22 g female ICR mice. The amount of neutralizing antibodies present in the serum of the immunized mice was determined using serum antibody titrations. The various serum dilutions (0.01 ml) were mixed with 5 LD₅₀ units of *C. botulinum* type E toxin and the mixtures were injected IP into mice. The neutralizations were performed in duplicate. The mice were then observed for signs of botulism for 4 days. Undiluted serum from day 28 did not protect, while undiluted, 1/10 diluted and 1/100 diluted day 70 serum protected (100% of animals) while 1/1000 diluted day 70 serum did not. This corresponds to a neutralization titer of 50-500 IU/ml.

These results demonstrate that seroconversion occurred and neutralizing antibodies were induced when the recombinant BotE protein was utilized as the immunogen.

EXAMPLE 43

5

Construction Of Vectors To Facilitate Expression Of His-Tagged BotE Protein In Fermentation Cultures

10

A number of expression vectors were constructed to facilitate the expression of recombinant BotE protein in large scale fermentation culture. These constructs varied as to the strength of the promoter utilized (T7 or T7lac) and the presence of repressor elements (lacIq) on the plasmid. The resulting constructs varied in the level of expression achieved and in plasmid stability which facilitated the selection of a optimal expression system for fermentation scaleup. This example involved a) construction of BotE expression vectors and b) determination of expression levels in small scale cultures.

15

a) Construction Of BotE Expression Vectors

The BotE expression vectors created for fermentation culture were engineered to utilize the kanamycin rather than the ampicillin resistance gene, and contained either the T7 or T7lac promoter, with or without the lacIq gene for the reasons outlined in Example 30.

20

In all cases, the protein expressed by the various expression vectors is the pHisBotE protein described in Example 41, with the only differences between clones being the alteration of various regulatory elements. Using the designations outlined below, the pHisBotE clone (Ex. 41) is equivalent to pHisBotE amp T7lac.

**i) Construction Of pHisBotE kan lacIq
T7lac**

pHisBotE kan lacIq T7lac was constructed by inserting the *XbaI/HindIII* fragment of pHisBotE which contains the BotE gene sequences into *XbaI/HindIII*-cleaved pET24a vector. Proper construction was confirmed by restriction digestion.

ii) Construction Of pHisBotE kan T7

pHisBotE kan T7 was constructed by ligating the BotE-containing *XbaI/SapI* fragment of pHisBotE kan lacIqT7lac to the T7 promoter-containing *XbaI/SapI* fragment of pET23a. Proper construction was confirmed by restriction digestion.

iii) Construction Of pHisBotE kan lacIqT7

pHisBotE kan lacIqT7 was constructed by inserting the *BglII/HindIII* fragment from pHisBotE kan T7 which contains the BotE gene sequences into *BglII/HindIII*-cleaved pET24 vector. Proper construction was confirmed by restriction digestion.

**b) Determination Of BotE Expression Levels In Small
Scale Cultures**

The three BotE kan expression vectors described above were transformed into BL21(DE3) competent cells and 50 ml (2XYT + 40 µg/ml kan) cultures were grown and induced with IPTG as described in Example 28. Total and soluble protein extracts from before and after induction made as described in Example 28. The total and soluble extracts were resolved on a 12.5% SDS-PAGE gel, and his-tagged proteins were detected on a Western blot utilizing the NiNTA-alkaline phosphatase conjugate as described in Example 31(c)(iii). The results showed that all three BotE cell lines expressed his-tagged proteins of the predicted MW for the BotE protein upon induction. The results also demonstrated that the two constructs that contained the T7 promoter expressed the BotE protein before induction, while the T7lac promoter construct did not. Upon induction, the T7 promoter-containing constructs induced to

higher levels than the T7lac-containing construct, with the pHisBotE kan lacIqT7/B121(DE3) cells accumulating the maximal levels of BotE protein.

EXAMPLE 44

Expression And Purification Of pHisBotE From Fermentation Cultures

5 Based on the small scale inductions performed in Example 43, the pHisBotE kan lacIq T7/B121(DE3) strain was selected for fermentation scaleup. This example involved the fermentation and purification of recombinant BotE C fragment protein.

 A fermentation with the pHisBotE kan lacIq T7/B121(DE3) strain was performed as described in Example 31. The fermentation culture was induced 2 hrs
10 post start of the glucose feed with 4 gm IPTG (final concentration = 1.6 mM). The OD₆₀₀ was 42 at time of induction, then 46.5, 48, 53 and 54 at 1-4 hrs post induction. Viable colony counts decreased from 0-4 hr induction [131, 4 (28), 7 (3), 7, 8; dilution 3 utilized 6 µl of dilution 2 cells; bracketed colonies are microcolonies]. All (32/32) colonies scored at the time of induction retained the BotE plasmid (kan resistant) and
15 no colonies at induction grew on IPTG+Kan plates (no mutations detected). These results were indicative of strong promoter induction, since colony viability reduced after induction, and the culture stopped growing during fermentation (stopped at 54 OD₆₀₀/ml).

 Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and total
20 protein was detected by staining with Coomassie blue. The results are shown in Figure 39.

 In Figure 39, lane 1 contains total protein from a pHisBotA kan T7 lac/B121(DE3) pLysS fermentation (Ex. 24). Lanes 2-9 contain extracts prepared from the above pHisBotE kan lacIq T7/B121(DE3) fermentation; lanes 2- 4 contain total
25 protein extracts prepared at 0, 1 and 2 hours post-induction, respectively. Lane 5 contains a soluble protein extract prepared at 2 hours post-induction. Lanes 6 and 7 contain total and soluble extracts prepared at 3 hours post-induction, respectively.

Lanes 8 and 9 contain total and soluble extracts prepared at 4 hours post-induction, respectively. Lane 10 contains broad range MW markers (BioRad).

The results shown in Figure 39 demonstrate that moderate level induction of totally soluble Bot E protein was observed, increasing from 1 to 4 hrs post induction (no expression was detected in uninduced cells). From a 2 liter fermentation harvest a 155 gm (wet wt) cell pellet was obtained and used to make a PEI-clarified lysate (1 liter in CRB, pH 6.8). The lysate was applied to a large scale IDA column and 200 mg of BotE protein, which was found to be greater than 95% pure (as judged by visual inspection of a Coomassie stained SDS-PAGE gel), was recovered. This represents 2.5% of the total soluble cellular protein (assuming a PEI lysate having a concentration of 8 mg protein/ml) and corresponds to a yield of 100 mg BotE protein/liter of fermentation culture.

The above results demonstrate that high levels of the recombinant BotE protein can be expressed and purified from fermentation cultures.

EXAMPLE 45

Removal Of Imidazole From Purified Recombinant BotE Protein Preparations

The expression of recombinant BotE protein, unlike the BotA and BotB proteins, did not require the presence of folding chaperones to maintain solubility during scale-up. A size exclusion chromatography step was included however to remove the imidazole from the sample and exchange the IDA elution buffer for one consistent with the BotA antigen.

A Sephacryl S-100 HR (S-100; Pharmacia) column was poured (2.5 cm x 24 cm; bed volume ~ 110 ml). Under these conditions, the BotE protein should be retained by the beads to a lesser degree than the smaller imidazole, therefore the BotE protein should elute from the column before the imidazole. The column was equilibrated in a buffer consisting of 50 mM sodium phosphate, 0.5 M NaCl, and 10% glycerol (all reagents from Mallinkrodt). Five milliliters of the IDA-purified BotE protein (Ex. 44) was filtered through a 0.45 μ syringe filter and applied to the S-100

column, and equilibration buffer was pumped through the column at a flow rate of 1 ml/minute. Eluted proteins were monitored by absorbance at 280 nm, and collected either manually or with a fraction collector. Appropriate tubes were pooled if necessary, and the protein was quantitated by absorbance at 280 nm and/or BCA protein assay. The isolated peaks were then analyzed by native and/or SDS-PAGE to identify the protein(s) and evaluate the purity.

Figure 40 provides a representative chromatogram showing the purification of IDA-purified BotE on the S-100 column. Even though folding chaperones were not over-expressed with this antigen, a small amount of protein eluted at a time consistent with the folding chaperones expressed with BotA and BotB proteins (Gro) (see the first peak). The second peak in the chromatogram contained the BotE protein, and the third peak was presumably imidazole. This presumed imidazole peak was isolated in comparable levels in IDA-purified BotA and BotB protein preparations as well.

These results demonstrate that size exclusion chromatography can be used to remove imidazole and traces of contaminating high molecular weight proteins from IDA-purified BotE protein preparations.

The S-100-purified BotE protein was tested for endotoxin contamination using the LAL assay as described in Example 24. This preparation was found to contain 64 to 128 EU/mg recombinant protein and is therefore substantially free of endotoxin.

The S-100 purified BotE was mixed with purified preparations of BotA and BotB proteins and used to immunize mice; 5 μ g of each Bot protein was used per immunization and alum was included as an adjuvant. After two immunizations with this trivalent vaccine, the immunized mice were challenged with *C. botulinum* toxin. The immunized mice contained neutralizing antibodies sufficient to neutralize between 100,000 to 1,000,000 LD₅₀ of either toxin A or toxin B and between 1,000 to 10,000 LD₅₀ of toxin E. The titer of neutralizing antibodies directed against toxin E would be expected to increase following subsequent boosts with the vaccine. These results demonstrate that a trivalent vaccine containing recombinant BotA, BotB and BotE proteins provokes neutralizing antibodies.

EXAMPLE 46

Expression Of The C Fragment Of The *C. botulinum* Serotype C Toxin Gene And Generation Of Neutralizing Antibodies

5 The *C. botulinum* type C1 neurotoxin gene has been cloned and sequenced [Kimura *et al.* (1990) Biochem. Biophys. Res. Comm. 171:1304]. The nucleotide sequence of the toxin gene derived from the *C. botulinum* type C strain C-Stockholm is available from the EMBL/GenBank sequence data banks under the accession number D90210; the nucleotide sequence of the coding region is listed in SEQ ID NO:59. The amino acid sequence of the *C. botulinum* type C1 neurotoxin derived from this strain
10 is listed in SEQ ID NO:60.

The DNA sequence encoding the native *C. botulinum* serotype C1 C fragment gene derived from the C-Stockholm strain can be expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:61 and the corresponding amino acid sequence is listed in SEQ ID NO:62. The C fragment region from any strain of
15 *C. botulinum* serotype C can be amplified and expressed using the approach illustrated below using the C fragment derived from *C. botulinum* type C C-Stockholm strain. Expression of the C fragment of *C. botulinum* type C1 toxin in heterologous hosts (*e.g.*, *E. coli*) has not been previously reported.

20 The C fragment of the *C. botulinum* serotype C1 (BotC1) toxin gene is cloned using the protocols and conditions described in Example 28 for the isolation of the native BotA gene. A number of *C. botulinum* serotype C strains (expressing either or both C1 and C2 toxin) are available from the ATCC [*e.g.*, 2220 (ATCC 17782), 2239 (ATCC 17783), 2223 (ATCC 17784; a type C- β strain; C- β strains produce C2 toxin), 662 (ATCC 17849; a type C- α strain; C- α strains produce mainly C1 toxin and a
25 small amount of C2 toxin), 2021 (ATCC 17850; a type C- α strain) and VPI 3803 (ATCC 25766)]. Alternatively, other type C strains may be employed for the isolation of sequences encoding the C fragment of *C. botulinum* serotype C toxin.

The following primer pair is used to amplify the BotC gene: 5'-CGCCATGGC
TTTATTAAAAGATATAATTAATG-3' [5' primer, engineered *Nco*I site underlined
(SEQ ID NO:63)] and 5'-GCAAGCTTTTATTCACTTACAGGTAC AAAACC-3' [3'
primer, engineered *Hind*III site underlined, native gene termination codon italicized
(SEQ ID NO:64)]. Following PCR amplification, the PCR product is inserted into the
pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as
described for BotA C fragment gene in Example 28. The resulting construct is termed
pHisBotC. Proper construction is confirmed by DNA sequencing of the BotC
sequences contained within pHisBotC.

pHisBotC expresses the BotC gene sequences under the transcriptional control
of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag
affinity tag. The pHisBotC expression construct is transformed into BL21(DE3)
pLysS competent cells and 1 liter cultures are grown, induced and his-tagged proteins
are purified utilizing a NiNTA resin (eluted in 250 mM imidazole, 20% glycerol) as
described in Example 28. Total, soluble and purified proteins are resolved by
SDS-PAGE and detected by Coomassie staining and Western blot hybridization
utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen) which recognizes
his-tagged proteins as described in Example 31(c)(iii). This analysis permits the
determination of expression levels of the pHisBotC protein (*i.e.*, number of mg/liter
expressed as a soluble protein). The purified BotC protein will migrate as a single
band of the predicted MW (*i.e.*, ~50kD).

The level of expression of the pHisBotC protein may be modified (increased)
by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the *lacIq*
gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in
fermentation cultures as described in Example 30. If only very low levels (*i.e.*, less
than 0.5%) of soluble pHisBotC protein are expressed using the above expression
systems, the pHisBotC construct may be co-expressed with pACYCGro construct as
described in Example 32. In this case, the recombinant BotC protein may co-purify
with the folding chaperones. The contaminating chaperones may be removed as

described in Example 34. Preparations of purified pHisBotC protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

The purified pHisBotC protein is used to generate neutralizing antibodies. BALBc mice are immunized with the BotC protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotC antibodies to neutralize native *C. botulinum* type C toxin is demonstrated using the mouse-*C. botulinum* neutralization model described in Example 36.

EXAMPLE 47

Expression Of The C Fragment Of The *C. botulinum*

Serotype D Toxin Gene And Generation Of Neutralizing Antibodies

The *C. botulinum* type D neurotoxin gene has been cloned and sequenced [Sunagawa et al. (1992) J. Vet. Med. Sci. 54:905 and Binz *et al.* (1990) Nucleic Acids Res. 18:5556]. The nucleotide sequence of the toxin gene derived from the CB16 strain is available from the EMBL/GenBank sequence data banks under the accession number S49407; the nucleotide sequence of the coding region is listed in SEQ ID NO:65. The amino acid sequence of the *C. botulinum* type D neurotoxin derived from the CB16 strain is listed in SEQ ID NO:66.

The DNA sequence encoding the native *C. botulinum* serotype D C fragment gene derived from a BotD expressing strain can be expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:67 and the corresponding amino acid sequence is listed in SEQ ID NO:68. The C fragment region from any strain of *C. botulinum* serotype D can be amplified and expressed using the approach illustrated below using the C fragment derived from *C. botulinum* type D CB16 strain. Expression of the C fragment of *C. botulinum* type D toxin in heterologous hosts (*e.g.*, *E. coli*) has not been previously reported.

The C fragment of the *C. botulinum* serotype D (BotD) toxin gene is cloned using the protocols and conditions described in Example 28 for the isolation of the

native BotA gene. A number of *C. botulinum* type D strains are available from the ATCC [*e.g.*, ATCC 9633, 2023 (ATCC 17851), and VPI 5995 (ATCC 27517)].

The following primer pair is used to amplify the BotD gene: 5'-CGCCATGGC
TTTATTAAAAGATATAATTAATG-3' [5' primer, engineered *Nco*I site underlined
(SEQ ID NO:63)] and 5'-GCAAGCTTTTACTCTACCCATCCTGGATCCCT-3' [3'
primer, engineered *Hind*III site underlined, native gene termination codon italicized
(SEQ ID NO:69)]. Following PCR amplification, the PCR product is inserted into the
pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as
described for BotA C fragment gene in Example 28. The resulting construct is termed
pHisBotD.

pHisBotD expresses the BotD gene sequences under the transcriptional control
of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag
affinity tag. The pHisBotD expression construct is transformed into BL21(DE3)
pLysS competent cells and 1 liter cultures are grown, induced and his-tagged proteins
are purified utilizing a NiNTA resin as described in Example 28. Total, soluble and
purified proteins are resolved by SDS-PAGE and detected by Coomassie staining and
Western blot hybridization utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen)
which recognizes his-tagged proteins as described in Example 31(c)(iii). This analysis
permits the determination of expression levels of the pHisBotD protein (*i.e.*, number of
mg/liter expressed as a soluble protein). The purified BotD protein will migrate as a
single band of the predicted MW (*i.e.*, ~50kD).

The level of expression of the pHisBotD protein may be modified (increased)
by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the *lacIq*
gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in
fermentation cultures as described in Example 30. If only very low levels (*i.e.*, less
than about 0.5%) of soluble pHisBotD protein are expressed using the above
expression systems, the pHisBotD construct may be co-expressed with pACYCGro
construct as described in Example 32. In this case, the recombinant BotD protein may
co-purify with the folding chaperones. The contaminating chaperones may be removed

as described in Example 34. Preparations of purified pHisBotD protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

The purified pHisBotD protein is used to generate neutralizing antibodies. BALBc mice are immunized with the BotD protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotD antibodies to neutralize native *C. botulinum* type D toxin is demonstrated using the mouse-*C. botulinum* neutralization model described in Example 36.

EXAMPLE 48

Expression Of The C Fragment Of The *C. botulinum*

Serotype F Toxin Gene And Generation Of Neutralizing Antibodies

The *C. botulinum* type F neurotoxin gene has been cloned and sequenced [East *et al.* (1992) FEMS Microbiol. Lett. 96:225]. The nucleotide sequence of the toxin gene derived from the 202F strain (ATCC 23387) is available from the EMBL/GenBank sequence data banks under the accession number M92906; the nucleotide sequence of the coding region is listed in SEQ ID NO:70. The amino acid sequence of the *C. botulinum* type F neurotoxin derived from the 202F strain is listed in SEQ ID NO:71.

The DNA sequence encoding the native *C. botulinum* serotype F C fragment gene derived from the 202F strain can be expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:72 and the corresponding amino acid sequence is listed in SEQ ID NO:73. The C fragment region from any strain of *C. botulinum* serotype F can be amplified and expressed using the approach illustrated below using the C fragment derived from *C. botulinum* type F 202F strain. Expression of the C fragment of *C. botulinum* type F toxin in heterologous hosts (*e.g.*, *E. coli*) has not been previously reported.

The C fragment of the *C. botulinum* serotype F (BotF) toxin gene is cloned using the protocols and conditions described in Example 28 for the isolation of the

native BotA gene. The *C. botulinum* type F 202F strain is obtained from the American Type Culture Collection (ATCC 23387). Alternatively, sequences encoding the BotF toxin may be isolated from any BotF expressing strain [e.g., VPI 4404 (ATCC 25764), VPI 2382 (ATCC 27321) and Langeland (ATCC 35415)].

5 The following primer pair is used to amplify the BotF gene: 5'-CGCCATGGC
TATTCTAATTATATATTTTAATAG-3' [5' primer, engineered *Nco*I site underlined
(SEQ ID NO:74)] and 5'-GCAAGCTTTCATTCTTCCATCCATTCTC-3' [3' primer,
engineered *Hind*III site underlined, native gene termination codon italicized (SEQ ID
NO:75)]. Following PCR amplification, the PCR product is inserted into the
10 pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as
described for BotA C fragment gene in Example 28. The resulting construct is termed
pHisBotF.

pHisBotF expresses the BotF gene sequences under the transcriptional control
of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag
15 affinity tag. The pHisBotF expression construct is transformed into BL21(DE3) pLysS
competent cells and 1 liter cultures are grown, induced and his-tagged proteins are
purified utilizing a NiNTA resin as described in Example 28. Total, soluble and
purified proteins are resolved by SDS-PAGE and detected by Coomassie staining and
Western blot hybridization utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen)
20 which recognizes his-tagged proteins as described in Example 31(c)(iii). This analysis
permits the determination of expression levels of the pHisBotF protein (i.e., number of
mg/liter expressed as a soluble protein). The purified BotF protein will migrate as a
single band of the predicted MW (i.e., ~50kD).

The level of expression of the pHisBotF protein may be modified (increased)
25 by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the lacIq
gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in
fermentation cultures as described in Example 30. If only very low levels (i.e., less
than about 0.5%) of soluble pHisBotF protein are expressed using the above expression
systems, the pHisBotF construct may be co-expressed with pACYCGro construct as

The C fragment of the *C. botulinum* serotype G (BotG) toxin gene is cloned using the protocols and conditions described in Example 28 for the isolation of the native BotA gene. The *C. botulinum* type G 113/30 strain is obtained from the NCFB. The following primer pair is used to amplify the BotG gene: 5'-CGCCATGGCTGAC
5 ACAATTTTAATACA AGT-3' [5' primer, engineered *Nco*I site underlined (SEQ ID NO:80)] and 5'-GCCTCGAGTTATTCTGTCCATCCTTCATCCAC-3' [3' primer, engineered *Xho*I site underlined, native gene termination codon italicized (SEQ ID NO:81)]. Following PCR amplification, the PCR product is inserted into the pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as
10 described for BotA C fragment gene in Example 28 with the exception that the sequences encoding BotG are excised from the pCRscript vector by digestion with *Nco*I and *Xho*I and the *Nco*I site is blunted (the BotG sequences contain an internal *Hind*III site). This *Nco*I(filled)/*Xho*I fragment is then ligated to the pETHisb vector which has been digested with *Nhe*I and *Sal*I and the *Nhe*I site is blunted. The
15 resulting construct is termed pHisBotG.

pHisBotG expresses the BotG gene sequences under the transcriptional control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag. The pHisBotG expression construct is transformed into BL21(DE3) pLysS competent cells and 1 liter cultures are grown, induced and his-tagged proteins
20 are purified utilizing a NiNTA resin as described in Example 28. Total, soluble and purified proteins are resolved by SDS-PAGE and detected by Coomassie staining and Western blot hybridization utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen) which recognizes his-tagged proteins as described in Example 31(c)(iii). This analysis permits the determination of expression levels of the pHisBotG protein (*i.e.*, number of
25 mg/liter expressed as a soluble protein). The purified BotG protein will migrate as a single band of the predicted MW (*i.e.*, ~50kD).

The level of expression of the pHisBotG protein may be modified (increased) by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the *lacIq* gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in

fermentation cultures as described in Example 30. If only very low levels (*i.e.*, less than about 0.5%) of soluble pHisBotG protein are expressed using the above expression systems, the pHisBotG construct may be co-expressed with pACYCGro construct as described in Example 32. In this case, the recombinant BotG protein may co-purify with the folding chaperones. The contaminating chaperones may be removed as described in Example 34. Preparations of purified pHisBotG protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

The purified pHisBotG protein is used to generate neutralizing antibodies. BALBc mice are immunized with the BotG protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotG antibodies to neutralize native *C. botulinum* type G toxin is demonstrated using the mouse-*C. botulinum* neutralization model described in Example 36.

EXAMPLE 50

Expression Of Recombinant Botulinal Toxin Proteins In Eucaryotic Host Cells

Recombinant botulinal C fragment proteins may be expressed in eucaryotic host cells, such as yeast and insect cells.

a) Expression In Yeast

Botulinal C fragments derived from serotypes A, B, C, D, E, F and G may be expressed in yeast cells using a variety of commercially available vectors. For example, the pPIC3K and pPIC9K expression vectors (Invitrogen) may be employed for expression in the methylotrophic yeast, *Pichia pastoris*. When the pPIC3K vector is employed, expression of the botulinal C fragment protein will be intracellular. When the pPIC9K vector is employed, the botulinal C fragment protein will be secreted (the alpha factor secretion signal is provided on the pPIC9K vector).

DNA sequences encoding the desired C fragment is inserted into these vectors using techniques known to the art. Briefly, the desired botulinal expression cassette (including sequences encoding the his-tag; described in the preceding examples) is

amplified using the PCR in conjunction with primers that incorporate unique restriction sites at the termini of the amplified fragment. Suitable restriction enzyme sites include *Sna*BI, *Eco*RI, *Avr*II and *Not*I. When the botulinal C fragment is to be expressed using the pPIC3K vector, the initiator methionine (ATG) is provided by the desired Bot gene sequence and a Kozak consensus sequence is engineered upstream of the ATG (*e.g.*, ACCATGG).

The amplified restriction fragment containing the botulinal C fragment gene is then cloned into the desired expression vector. Recombinant clones are integrated into the *Pichia pastoris* genome and recombinant protein expression is induced using methanol following the manufacturer's instructions (Invitrogen *Pichia* expression kit manual).

C. botulinum genes are A/T rich and contain multiple sequences that are similar to yeast transcriptional termination signals (*e.g.*, TTTTATA). If premature transcription termination is observed when the botulinal C fragment genes are expressed in yeast, the transcription termination signals present in the C fragment genes can be removed by either site directed mutagenesis (utilizing the pALTER system; Promega) or by construction of synthetic genes utilizing overlapping synthetic primers.

The botulinal C fragment genes may be expressed in other yeast cells using other commercially available vectors [*e.g.*, using the pYES2 vector (Invitrogen) and *S. cerevisiae* cells (Invitrogen)].

b) Expression In Insect Cells

Botulinal C fragments derived from serotypes A, B, C, D, E, F and G may be expressed in insect cells using a variety of commercially available vectors. For example, the pBlueBac4 transfer vector (Invitrogen) may be employed for expression in *Spodoptera frugiperda* (Sf9) insect cells (baculovirus expression system) (equivalent baculovirus vectors and host cells are available from other vendors, *e.g.*, Pharmingen, San Diego, CA). Botulinal C fragments contained on *Nco*I/*Hind*III fragments

contained within the pHisBotA-G expression constructs (described in the preceding examples) are cloned into the pBlueBac4 vector (digested with *NcoI* and *HindIII*); the *NcoI* site present on the C fragment constructs overlaps with the start codon of the fusion proteins. In the case of botulinal C fragment clones that contain internal
5 *HindIII* sites (e.g., using the BotG sequences described in Ex. 49), the C fragment gene is contained within a *NcoI/XhoI* fragment on the pHisBot construct. This *NcoI/XhoI* fragment is excised from pHisBot and inserted into pBlueBac4 digested with *NcoI* and *SaII*. Recombinant baculoviruses are made and the desired recombinant C fragment is expressed in *Sf9* cells using the protocols provided by the manufacturer
10 (Invitrogen MaxBac manual). The resulting constructs will express the pHisBot protein intracellularly (including the N-terminal his-tag) under the control of the polyhedrin promoter. For extracellular secretion of botulinal C fragment proteins, the C fragment sequences from the pHisBot constructs are cloned into the pMelBacB vector (Invitrogen) as described above for the pBlueBac4 vector. When the pMelBacB
15 vector is employed, the his-tagged botulinal C fragment proteins are secreted (utilizing a vector-encoded honeybee melittin secretion signal) and contain a nine amino acid extension at the N-terminus.

His-tagged botulinal C fragments expressed in yeast or insect cells are purified using metal chelation columns as described in the preceding examples.

20 From the above it is clear that the present invention provides compositions and methods for the preparation of effective multivalent vaccines against *C. botulinum* neurotoxin. It is also contemplated that the recombinant botulinal proteins be used for the production of antitoxins. All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and
25 variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention.